DEVELOPMENT OF IMMUNOTHERAPIES AND VACCINES AGAINST VISCERAL LEISHMANIASIS.

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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature:

Date: 04 JAN 2016

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Abstract

Visceral leishmaniasis (VL) is a chronic parasitic disease prevalent in tropical and sub-tropical countries caused by the protozoan parasites *Leishmania donovani* and *Leishmania infantum* (*chagasi*). VL is associated with severe immune dysfunction and clinical outcomes of infection depend on the infecting parasite species and the host immune response. Immunity against invading pathogens requires strong innate and adaptive host immune responses, but *Leishmania* parasites can elude these defence mechanisms to persist and survive in the host. Treatment options are limited to relatively toxic drugs and no vaccine for humans is available. Identifying and understanding the host immune responses is of paramount importance to better understand disease pathogenesis and for the development of vaccines and therapies. This study has focused on the development of immune-based therapy with immune checkpoint inhibitors and/or activators, as well as cytokines as a way to treat disease either alone or in combination with conventional drugs. In addition, I developed a platform for a live attenuated whole parasite vaccine against experimental VL.

The first aim of this study focused on combination immunotherapy as a way to treat VL either alone or with conventional drugs. Previous studies have shown that activation of glucocorticoid-induced TNF receptor family-related protein (GITR) in *L. donovani* -infected mice boosted $CD4^+$ T cell activation and reduced liver parasite burden. Similarly, IL-10 blockade has previously been shown to enhance host resistance against *L. donovani*. I investigated whether combined GITR stimulation and IL-10 blockade would act synergistically to improve anti-parasitic immunity in mice infected with *L. donovani*. Infected mice were treated with a combination of an agonist anti-GITR mAb and a blocking anti-IL-

10R mAb, and parasite burdens were assessed. Mice treated with this combination did not control parasite growth any better than mice treated with a single form of immune modulation. However, combination immune therapy in mice infected with a low dose of parasites was detrimental, similar to what has been observed in humans, while no such effect was seen in mice with high parasite burdens. Nevertheless, combined anti-IL-10 and anti-GITR mAb treatment could improve anti-parasitic immunity when used with sub-optimal doses of anti-parasitic drug. These results have implications for the use of immune therapies in patients, and suggest that the outcomes may differ depending on the stage of disease, the immune modulators used and use of anti-parasitic drug.

The second aim of the study focused on the use of cytokine therapy, by testing the effect of IL-2/anti-IL-2 mAb complexes to treat experimental VL. IL-2/anti-IL-2 mAb complexes have significant effects on the immune system, and have been studied extensively in various disease settings, including cancer treatment and various infections. However, the impact of IL-2/anti-IL-2 mAb complex treatment on *L. donovani* infection has not been previously investigated. In my study, two doses of the IL-2/anti-IL-2 mAb complexes (IL-2Jc or the IL-2Sc) resulted in a significant reduction in parasite burdens in mice infected with *L. donovani*. However, no expansion of targeted cell populations was observed, as previously reported. Further investigations with transgenic mice and cell depleting antibodies revealed that $CD4^+$ T cell were required for the maintenance of anti-parasitic immunity generated by the IL-2/anti-IL-2 mAb complex treatments. This study has therefore provided evidence for the efficacy of cytokine-based IL-2/anti-IL-2 mAb complex therapy for treating VL and highlights that timing and dose of treatment should be considered carefully before treating.

The final aim of the study focused on developing a live attenuated, whole parasite vaccine to protect against experimental VL. I evaluated the potential of both irradiation and chemical attenuation of *L. donovani* parasites as a vaccine strategy. *L. donovani* amastigotes or *in-vitro* cultured promastigotes were irradiated at 500 Gys or treated with tafurmycin, an alkylating agent that irreversibly alters the parasite DNA, thus inhibiting parasite growth. I found that irradiated *L. donovani* promastigotes provided better protection compared to irradiated amastigotes. However, irradiated parasites were still able to expand in immunocompromised animals, while this did not appear to be the case for chemically attenuated parasites. Furthermore, addition of adjuvants CpG-DNA or Poly (I:C) did not further improve vaccine mediated protection. Although this vaccine has not yet been optimised, it did generate potent anti-parasitic $CD4^+$ T cell responses and reduced parasite burdens in infected tissue sites. Since many chronic infectious diseases share mechanisms of immune suppression, these findings may have broader implications for other infectious diseases, such as HIV, tuberculosis and malaria.

Keywords

$CD4^+$ T cells, *Leishmania*, liver, spleen, immune therapy, vaccines, visceral leishmaniasis,

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Publications

Published work by the author incorporated into the thesis:

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1. Immune regulation during chronic visceral leishmaniasis. **Faleiro RJ**, Kumar R, Hafner LM, Engwerda CR. PLoS Negl Trop Dis. 2014 Jul 10;8(7). Noting that parts of this review have been incorporated into Chapter 2 (Literature Review) which also includes updated material for the thesis that is mot found in the review.

In preparation:

1. Combination immune-therapy for the treatment of visceral leishmaniasis.
2. IL-2/anti-IL-2 complexes mediate protection against *L. donovani* through $CD4^+$ T cells.

Poster Presentation

1. **Rebecca Faleiro**; Rajiv Kumar; Louise Hafner; Christian Engwerda. Modulating $CD4^+$ T cells during chronic Visceral Leishmaniasis (VL) to improve disease outcome. Keystone Symposia on "T Cells: Regulation and Effector Function". Utah, USA. March 2015.

Additional published work by the author relevant to the thesis but not forming part of it:

1. Tissue requirements for establishing long-term $CD4^+$ T cell-mediated immunity following *Leishmania donovani* infection. Bunn PT, Stanley AC, de Labastida Rivera F, Mulherin A, Sheel M, Alexander CE, **Faleiro RJ**, Amante FH, Montes De Oca M, Best SE, James KR, Kaye PM, Haque A, Engwerda CR. *J Immunol.* 2014 Apr 15;192 (8):3709-18.

List of Abbreviations

Ab	Antibody
AIDS	Acquired Immune Deficiency Syndrome
AMC	Age matched control
APC	Antigen Presenting Cell
BM	Bone Marrow
BSA	Bovine Serum Albumin
CBA	Cytometric Bead Array
CCL	(C-C motif) Ligand
CD	Cluster of Differentiation
CL	Cutaneous Leishmaniais
CMI	Cell Mediated Immunity
CO_2	Carbon Dioxide
CpG ODN	CpG Oligodeoxynucleotides
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
CXC	C-X-C chemokines
CXCL	(C-X-C motif) Ligand
CXCR	(C-X-C motif) Receptor
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide
DT	Diphtheria Toxin

DTR	Diphtheria Toxin Receptor
EDTA	Ethylenediaminetetraacetic Acid
EVL	Experimental Visceral Leishmaniasis
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
Foxp3	Forkhead Box P3
g	Gram
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-Induced TNFR Family related gene)
Gy	Gray
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HLA	Human Leucocyte Antigen
Hrs	Hours
i.m.	Intramuscularly
i.p.	Intraperitoneally
i.v.	Intravenously (i.v.)
iDTR	Inducible Diphtheria Toxin Receptor
IFNγ	Interferon Gamma
IL	Interleukin
ISO	Isotype Control
KC	Kupffer cells
KLRG-1	Killer-cell Lectin like Receptor G1
LDU	Leishmania Donovan Units
M199	Medium 199

mAb	Monoclonal Antibody
MACS	Magnetic-Activated Cell Sorting
mg	Milligrams
mL	Millilitre
MLR	Mixed Lymphocyte Reaction
MM	Marginal Metallophilic
mM	Millimolar
MMM	Marginal Metallophillic Macrophages
MNCs	Mononuclear Cells
MO	Monocyte
mRNA	Messenger RNA
MZ	Marginal Zone
MZM	Marginal Zone Macrophages
Mφ	Macrophage
n	No of Mice
NaCl	Sodium Chloride
ng	Nanogram
NK	Natural Killer cell
NKp46	Natural killer cell p46-related protein]
NO	Nitric Oxide
NO_2^-	Nitrite
NRAMP	Natural Resistance-Associated Macrophage Protein
p.c.	Post Challenge
pg	Pico grams
p.i.	Post Infection

PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Saline Buffer
PCR	Polymerase Chain Reaction
PD-1	Programmed Death -1
PD-L1	Programmed Death -1 Ligand -1
PFA	Paraformaldehyde
PKDL	Post kala-azar dermal leishmaniasis
PMA	Phorbol 12-Myristate 13-Acetate
Poly I:C	Polyinosinic-polycytidylic acid
PS	Penicillin/Streptomycin
RAG	Recombination-Activating Gene
RBC	Red blood cells
RNI	Reactive Nitrogen Intermediates
ROI	Reactive Oxygen Intermediates
RORγt	Retinoid-Acid Receptor-related Orphan Receptor
RPMI	Roswell Park Memorial Institute medium
RT	Room Temperature
Sb^v	Pentavalent Antimonial
SEM	Standard Error of Mean
SSG	Sodium Stibogluconate
Sub	Suboptimal
T-Bet	T-box Transcription Factor
TCR	T cell Receptor
TFA	Tafuramycin A
TGF-β	Transforming Growth Factor Beta

Th1	T Helper Cells
TNFα	Tumour Necrosis Factor alpha
Tr1	Type 1 Regulatory T cells
Treg	Regulator T cell
U	Units
VL	Visceral Leishmaniasis
µg	Microgram
µl	Microliter
µM	Micromole

Chapter 1: Introduction

1.1 DESCRIPTION OF SCIENTIFIC PROBLEM

Leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania*. It affects people and animals in many parts of the world, with higher incidence in the tropical and sub-tropical regions. There are about 21 known species of *Leishmania* that can cause the disease in humans [1]. Leishmaniasis can be broadly divided into visceral and cutaneous forms. The focus of my study is the visceralizing form, known as Visceral Leishmaniasis (VL).

VL is a fatal human disease if left untreated. The estimated incidence of VL is between 500,000 and 1,000,000 cases worldwide, and results in 20,000 – 40,0000 deaths each year [2] (Figure 1.1). Six main countries: India, Ethiopia, Bangladesh, Sudan, South Sudan and Brazil, account for 90 % of VL cases [2], and the above numbers are likely to be gross underestimates due to poor diagnosis and reporting.

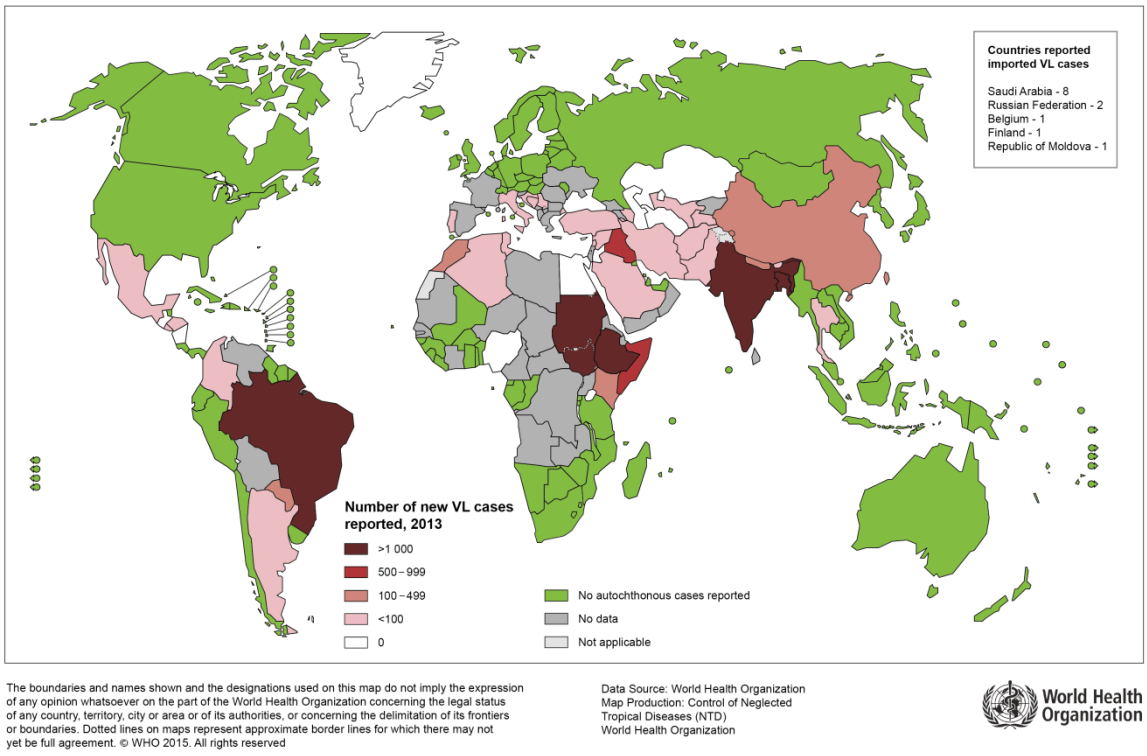

Figure 1.1: Current global distribution of Visceral Leishmaniasis.

(http://gamapserver.who.int/mapLibrary/Files/Maps/Leishmaniasis_2013_VL.png)

The most common treatment for VL has been pentavalent antimonials. However, there is now considerable parasite resistance against these drugs [3]. Newer drugs against VL have been developed in the recent years, but these are far from ideal because of their toxicity and cost [4].

At present there is no vaccine available against human leishmaniasis [5]. Studies have shown that immunization against leishmaniasis is achievable. “Leishmanisation”, which involves the process of deliberately infecting people with parasites causing cutaneous leishmaniais (CL), on hidden areas of the body, results in long term protection [6]. However despite the solid immunity that most immunized individuals develop, the practise was largely abandoned due to the development of complications such as large skin lesions, exacerbation

of skin diseases and poor responses to the vaccine [7, 8]. One of the major problems for developing vaccines to either prevent or treat VL has been the limited understanding of the immune mechanisms required for the control of parasite growth without causing disease. Our current understanding of the host immune response during VL largely arises from studies performed in *L. donovani* -infected, genetically susceptible mice.

1.2 **SIGNIFICANCE**

$CD4^+$ T cell responses are critical for effective immunity against *L. donovani* infection. This research aims to identify immune mechanisms that aid or hinder the development of protective $CD4^+$ T cell immune responses during VL. My research aims to identify targets for immune modulation that can be used to improve vaccines or as therapies. Results from my experiments will identify mechanisms of immune suppression of $CD4^+$ T cells responses in experimental VL, and this will have implications for helping to treat and/or prevent human VL. In addition, because many chronic infectious diseases share mechanisms of immune suppression, my findings may have broader implications for other infectious diseases, such as HIV, tuberculosis and malaria.

1.3 **HYPOTHESIS**

My hypothesis is that during an established VL infection, organ specific $CD4^+$ T cell responses that govern disease outcome are suboptimal due to increased immune regulatory activity that can be specifically targeted to improve disease outcome.

1.4 **AIMS**

To test the above hypothesis, I addressed the following aims:

1. Test whether promoting parasite-specific $CD4^+$ T cell function via GITR activation improves the outcome of experimental VL caused by *L. donovani.*
2. To test whether IL-2 signalling pathways are deficient in T cells during VL and to test the ability of IL-2/anti-IL-2 complexes to treat and improve experimental VL outcome.
3. To compare different methods of parasite attenuation and establish whether a live, attenuated, whole parasite vaccine can protect against VL.

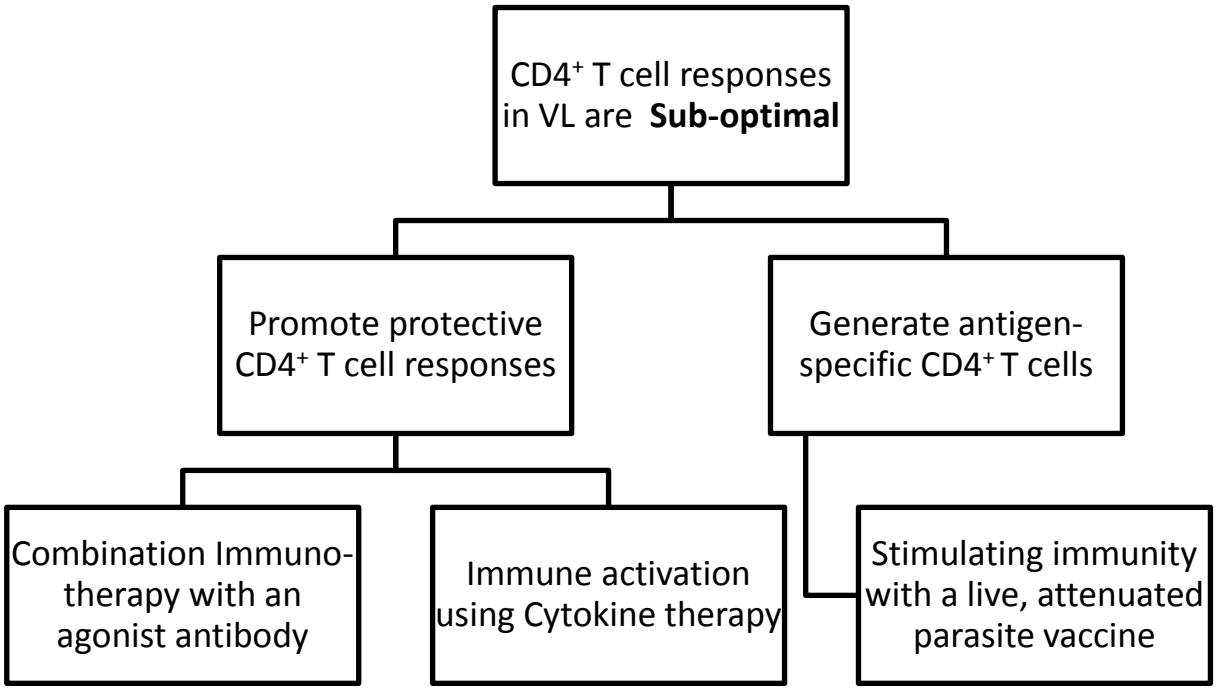

Figure 1.2 : **Graphical representation of Aims.**

Chapter 2: Literature Review

2.1 INTRODUCTION

Protozoan parasites belonging to the genus *Leishmania* are obligate intracellular parasites that are transmitted by the bite of the female Phlebotomine sand fly. The injected, flagellated metacyclic promastigote form is phagocytosed by host cell macrophages, in which they develop into the non-flagellated, replicative amastigote form [9]. Parasite numbers increase via binary fission, which ultimately results in the bursting of the cells, allowing the parasite to infect other phagocytic cells and continue its life cycle [10] (Figure 2.1).

There are 21 known species of *Leishmania* that can cause disease in humans, and these vary in virulence and infectivity [11]. The diseases caused by *Leishmania* parasites in humans include (a) **Cutaneous leishmaniasis (CL),** which is characterised by cutaneous/skin lesions which resolve in time, leaving noticeable scars. CL is caused by most *Leishmania* species capable of infecting humans. (b) **Mucocutaneous leishmaniasis (MCL),** which starts as a skin lesion, before spreading and causing progressive damage to tissue, especially to the areas of mucosal tissue in the mouth and nose. This disease is caused mainly by *L. braziliensis.* (c) **Diffuse cutaneous leishmaniasis (DCL)** is characterised by disfiguring skin lesions, which are often mistaken for lepromatous leprosy. It is caused by the species *L. aethiopica* or *L. amazonensis* [10] and is difficult to treat. (d) **Visceral leishmaniasis (VL)** is the most deadly form of the disease, and is caused by *L. donovani* and *L. infantum* (*chagasi*).

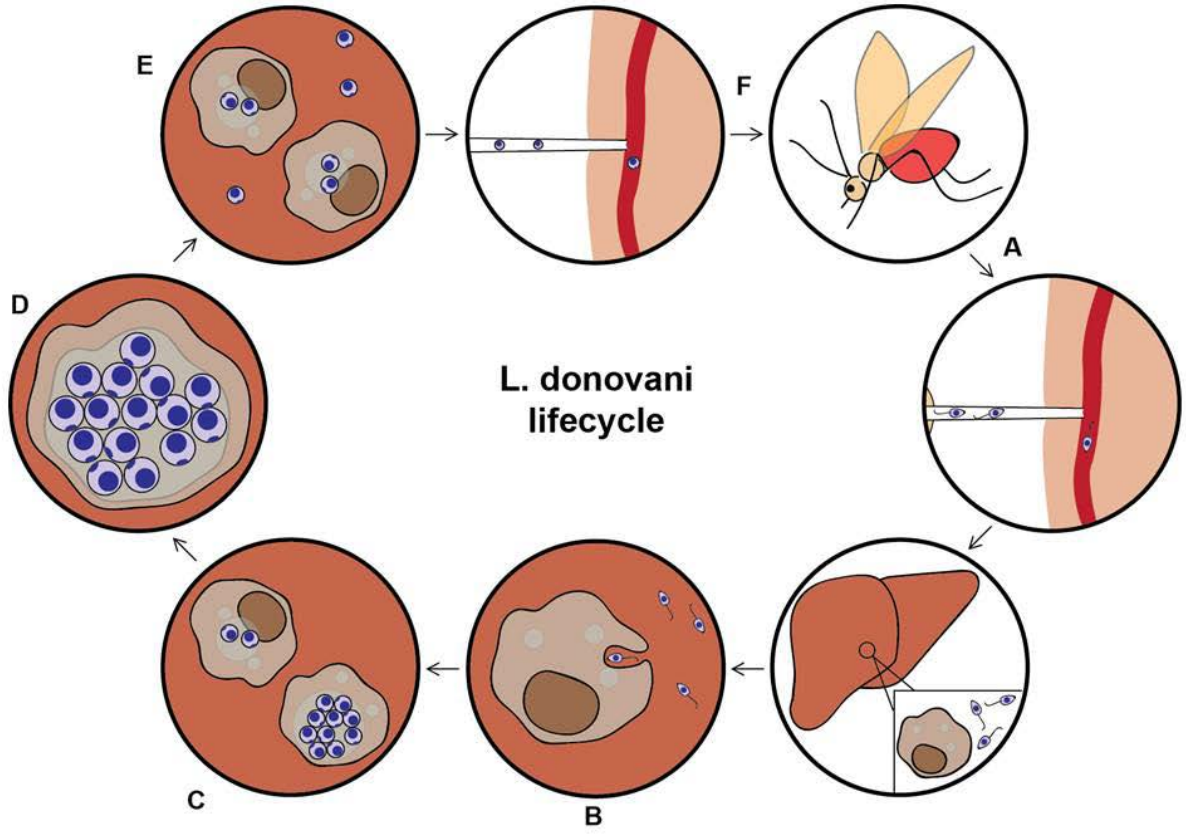

Figure 2.1: Life cycle of the *L. donovani* parasite.

When an infected Phlebotomine sand fly takes a blood meal (A) it transfers metacyclic promastigotes into a vertebrate host. (B) The parasites are phagocytosed by host macrophages and (C) change into amastigotes which multiply and (D) rupture the macrophage, (E) infecting neighbouring macrophages. (F) The life cycle is complete when infected macrophages are taken up by a female Phlebotomine sand fly when feeding on an infected host.

2.2 HUMAN VL

VL, also known as Kala-azar in the Indian sub-continent, is caused by *L. donovani* in humans and *L. infantum (chagasi)* in both humans and canines [12]. VL is the most fatal parasitic disease after malaria and affects hundreds of thousands of the world's poorest people in tropical countries. The clinical spectrum ranges from asymptomatic infection to fatal VL. The commonly targeted organs during VL are the bone marrow, liver and the spleen [13], while macrophages in the viscera are the main host cells for VL-causing parasites [14].

Clinical symptoms include splenomegaly and hepatomegaly, which results in an enlarged abdomen. Other symptoms include fever, muscle wasting, anaemia and weight loss [15, 16]. Hyper-pigmentation of warmer skin regions and the abdomen is commonly observed in Indian patients, hence the derivation of the name kala-azar, which means black fever in Hindi [17]. If left untreated, almost all symptomatic patients die within months of disease onset. The diagnosis of VL is confirmed by microscopic demonstration of amastigotes in spleen or bone marrow (BM) biopsies. Serological tests, such as rK39 dipsticks, are also used for diagnosis, but with the limitation that they cannot differentiate between past and present infection. PCR is another potential diagnostic option [18], but has not been validated for use in field settings where VL is endemic.

2.2.1 VL susceptibility

The majority of the people infected with *L. donovani* never develop VL [19-22]. The factors that influence susceptibility to VL are not fully understood. However, several genetic factors have been identified, including polymorphisms in the *NRAMP1/Slc11a1* gene [23, 24], but these appear to have no role in VL affecting the Indian population [25]. Polymorphisms in the CXCR2 gene, which encodes the receptor for IL-8 and other CXC chemokines, appears to plays a role in determining VL outcome in Indian patients [26]. Polymorphisms in the IL-2Rβ gene, which is involved in T cell activation, is also implicated in determining VL susceptibility [27]. However, polymorphisms in the HLA genes not only play roles in susceptibility to experimental VL [28-30], but a recent study has identified single nucleotide polymorphisms (SNPs) in this gene region that are strongly associated with both resistance and susceptibility to VL in Indian and Brazilian populations [31]. Nutritional

status can also influence disease susceptibility, with malnutrition being a major risk factor for VL, especially in rural settings [32]. Malnutrition negatively impacts on both cell-mediated and innate immunity [33, 34]. In addition, helminth infections are very common in rural, VL-endemic areas, which may favour *Leishmania* parasite replication [35, 36]. Other epidemiological factors, such as living proximity to a previous VL patient, are also risk factors for developing VL [37].

2.2.2 Disease spectrum of human VL

Unlike experimental VL, where there is a well-defined organ-specific course of infection, human VL manifests as a more heterogeneous form of disease with different levels of chronic infection observed in the spleen, liver and BM [38]. Following the course of the infection in VL patients requires invasive techniques such as spleen and BM aspiration, which are uncomfortable, potentially dangerous and time consuming. However, because a better understanding of the range of disease manifestations in human VL is required and may assist in understanding disease pathogenesis, these methods are employed in various research projects as part of routine diagnostic procedures.

Most human *Leishmania* infections are subclinical or asymptomatic and this can be attributed to the development of effective anti-parasitic, cell mediated immune responses [39, 40]. Only a small proportion of infected individuals develop disease, and VL patients that recover from infection are usually resistant to re-infection [40, 41]. Depressed cell mediated immunity is a characteristic of human VL and is observed by a negative *Leishmania* skin test and the failure of peripheral blood mononuclear cells (PBMCs) to proliferate and produce

interferon gamma (IFNγ) in response to *Leishmania* antigen [42]. In contrast, PBMCs taken from patients cured of VL are able to proliferate and produce IFNγ and tumour necrosis factor alpha (TNFα) [42], suggesting that T cell responses in VL patients are refractory to antigenic stimulation [43]. However, recent studies carried out showed that whole blood cells taken from active VL patients and stimulated with parasite antigen were able to produce elevated and similar levels of IFNγ as observed in cured VL patients, indicating that antigen-specific T cells were not refractory to stimulation, but rather, other immunosuppressive factors might contribute to unfavourable clinical outcomes [42, 44, 45]. They also showed that significant amounts of interleukin-10 (IL-10) were produced by whole blood cells from VL patients in response to stimulation with parasite antigens in whole blood assays [42, 44].

2.2.3 Immune regulation during human VL

Although VL initially was thought to be associated with a Th2 dominated immune response, indicated by elevated levels of IL-4 and/or IL-13 [46, 47], more recent studies indicate that there is not a clear Th2 bias in human VL. Typically, VL is associated with increased production of multiple pro-inflammatory cytokines and chemokines. VL patients have elevated plasma protein levels of IL-1, IL-6, IL-8, IL-12, IL-15, IFNγ inducible protein-10 (IP-10), monokine induced by IFNγ (MIG), IFNγ and TNFα [46, 48]. Elevated levels of IFNγ mRNA have been found in the spleen and bone marrow during the acute phase of infection [46]. These observations suggest that unfavourable clinical outcomes are not related to Th2 skewing *per se*, but that other mechanisms contribute to VL pathogenesis.

Studies on clinical samples have shown that elevated levels of IL-10 correlate with increased incidence of several human chronic infectious diseases, such as HIV, tuberculosis (TB) and malaria [49-52]. As mentioned earlier, IL-10 is an important regulatory cytokine that suppresses potentially damaging inflammatory immune responses [53]. However, these immunosuppressive properties of IL-10 can also target antigen presentation pathways in macrophages and DC's, thereby affecting T cell activation and cytokine production during chronic infection, potentially promoting parasite persistence [53]. VL patients have elevated levels of IL-10 in serum, and IL-10 mRNA accumulation was increased, relative to controls, in BM and spleen tissue [42]. IL-10 blockade in *ex vivo* cell assays using spleen tissue from VL patients, showed increased IFNγ and TNFα production associated with significantly reduced parasite growth [54], indicating that IL-10 is a major suppressor of leishmanicidal immune mechanisms in human VL patients (Figure 2.3). Other IL-10 neutralizing studies also showed enhanced IFNγ production by antigen activated whole blood cells taken from VL patients [42]. A similar result was also found in studies on PBMCs from VL patients, where increased IFNγ production, as well as enhanced T cell proliferation, was observed following IL-10 blockade [55-57]. The IL-10 in these human samples appeared to be produced predominantly by highly activated IL-10-producing Th1 (Tr1) cells [46]. However, another study recently showed that regulatory T (Treg) cells accumulated in the BM of VL patients and were a source of IL-10 that could suppress anti-parasitic immunity [58].

Recent work by Ansari *et al.* showed elevated levels of circulating IL-27 and increased IL-27 mRNA accumulation in the spleen of VL patients, as well as enhanced expression of IL-21 mRNA [44]. IL-21 plays a role in amplifying IL-10 production by Tr1 cells induced by IL-27 [59]. The IL-27 and IL-21 in these samples appeared to be produced mainly by $CD14^+$ (monocytes/macrophages) cells and $CD3^+$ T cells, respectively [44]. Thus, these studies

support the notion that IL-27 and IL-21 are key cytokines that promote the differentiation and expansion of antigen specific IL-10 producing Tr1 cells during VL (Figure 2.3).

Human VL is also associated with high levels of plasma antibodies. Although sometimes useful in diagnosis, the role of antibodies in pathogenesis of VL is not clear. The high level of antibodies may drive the formation of immune complexes which can bind to the Fc receptors on macrophages leading to the production of IL-10 by macrophages [60], and thus contribute to VL pathogenesis. Another cytokine, TGF-β also has suppressive functions, and active VL is associated with increased plasma and mRNA levels of this cytokine [61]. The parasite-derived factor Cathepsin-B, present in *L. donovani,* can activate TGF-β, which then has the potential to negatively impact on macrophage activity by lowering reactive nitrogen intermediate (RNI) production [62, 63]. A better understanding of the precise mechanisms of TGF-β and IL-10 induction and activity during VL is required.

IL-17 has emerged as a potentially important cytokine in VL. A study in a Sudanese village during a VL outbreak over a 6 year period found that IL-17 and IL-22 production by PBMCs were tightly and independently associated with resistance to VL [64]. Thus, IL-17 and IL-22 may play complimentary roles to Th1 cytokines in controlling parasite growth and preventing the development of VL (Figure 2.2). The cellular mechanisms of parasite control induced by these cytokines remain unknown. Furthermore, the factors involved in regulating the production of these cytokines during active VL have not been fully elucidated, although IL-27 has been suggested to be involved in blocking Th17 expansion during infection [20]. Dissection of these processes should provide new insights into host control of parasite growth and resistance to VL.

The role of $CD4^+$ T cells and Treg cells in human VL has been widely studied, but data on the role of $CD8^+$ T cells is scarce. $CD8^+$ T cells, like $CD4^+$ cells, have immune regulatory capacity and can also directly kill the parasite infected macrophages through cytolytic enzymes such as granzymes, granulysin and perforin [65-67]. IL-10-producing $CD8^+$ T cells have been reported in human PKDL and *L. guanyensis* infection [68, 69], while a recent study has shown that $CD8^+$ T cells have an anergic or exhausted phenotype, as indicated by high expression of CTLA-4, PD-1 and IL-10, which may affect the protective capacity of these cells during clinical VL [70]. A better understanding of the role of $CD8^+$ T cells in VL may help to harness the anti-parasitic potential of these cells through vaccination or immune therapy.

The study of VL in humans can be difficult as it often requires an invasive form of tissue analysis, as the primary sites of infection are the spleen, liver and BM. Hence, our current understanding of the host immune responses during VL are largely based on studies carried out using a mouse model.

2.3 **THE MOUSE MODEL OF VL**

Studies in mice are carried out by establishing infection with an intravenous injections of *L. donovani* amastigotes into genetically susceptible mice [71]. Resistance and susceptibility to *L. donovani* infection in mice is controlled by the *Slc11a1* gene (formerly Nramp1- 'natural resistance associated macrophage protein 1') present in both mice and humans [72]. This gene is involved in the activation of macrophages during infectious disease. Genetically resistant mice have a functional *Slc11a1* gene, while susceptible mice

have a naturally occurring Glysine → Aspartic acid amino acid mutation, resulting in a non-functional *Slc11a1* gene [13]. BALB/c and C57BL/6 mice are genetically susceptible to *L. donovani* infection and are commonly used for experimental studies. *Leishmania* infection in these mice is non-fatal and immune-related tissue pathology observed in these animals show some similarity to those reported in clinical VL in humans [73].

In genetically susceptible mice infected with *L. donovani*, distinct organ-specific immune responses are observed as the disease progresses [74]. The liver is the site of an acute and resolving infection, whereas in the spleen and bone marrow (BM), a chronic infection becomes established [73, 75].

2.3.1 Establishment of infection in the Liver

In the liver during experimental VL in genetically susceptible mice, parasitic burdens peak between weeks 2-4 of infection and then resolve by weeks 6-8, although sterilising immunity is not achieved [12]. Clearing of the infection in the liver depends on the formation of inflammatory granulomas [76]. Following infection, the tissue macrophages in the liver, known as Kupffer cells (KC), are infected by amastigotes [77]. Early cytokine production by KC's plays a role in recruiting more monocytes and neutrophils to the site of infection that further amplify chemokine production [78, 79]. One study suggested that neutrophils play a protective role early during *L. donovani* infection [80]. However, there is also strong evidence from models of CL that these cells may help establish infections by acting as a safe-haven for parasites before being taken up by monocytes [81]. An important anti-parasitic role for monocytes in the early control of *L. donovani* infection has been established [78, 82],

although this may be more complicated than first thought, given the plasticity of these cells and their ability to differentiate into potent APC or regulatory cells [83, 84]. The recruitment of neutrophils and monocytes into the liver is followed by the recruitment of T cells, that are critical for efficient granuloma formation around infected KC and control of parasite growth [85]. In particular, activation of T cells via interleukin (IL-12) leads to their recruitment and production of the pro-inflammatory cytokines IFNγ and TNFα [86], which further amplifies cellular recruitment around infected KCs [13], and also activates anti-microbial mechanisms in these cells [13]. These microbicidal mechanisms include the generation of reactive oxygen intermediates (ROI) and RNI, that are both capable of killing parasites in infected macrophages, although only the latter is critically important for the resolution of the disease [87] (Figure 2.2). Recent studies have identified the C-type lectin receptors Dectin-1, mannose receptor and specific intercellular adhesion molecule-3-grabbing non-integrin receptor 3 (SIGNR3; a homologue of human DC-SIGN), as important pattern recognition receptors for *L. infantum* [88], and also showed that early inflammasome-derived IL-1β is critical for the induction of RNI by *L. infantum*-infected macrophages [89], thus identifying critical early events in parasite recognition and control by the host.

After 4 weeks of *L. donovani* infection, well organised and functionally mature granulomas are observed in the liver, associated with the control of parasite growth and a decline in parasite burden [90]. Parasite numbers decline until 6-8 weeks post-infection, after which, a relatively low-level persistent infection becomes established that is contained within granulomas by $CD4^+$ T cells [91, 92]. Following re-infection, parasite growth is controlled within 1-2 weeks, with parasite burden only reaching a fraction of the primary infection, indicating the development of productive, concomitant immunological memory that may include a $CD8^+$ T cell component [77].

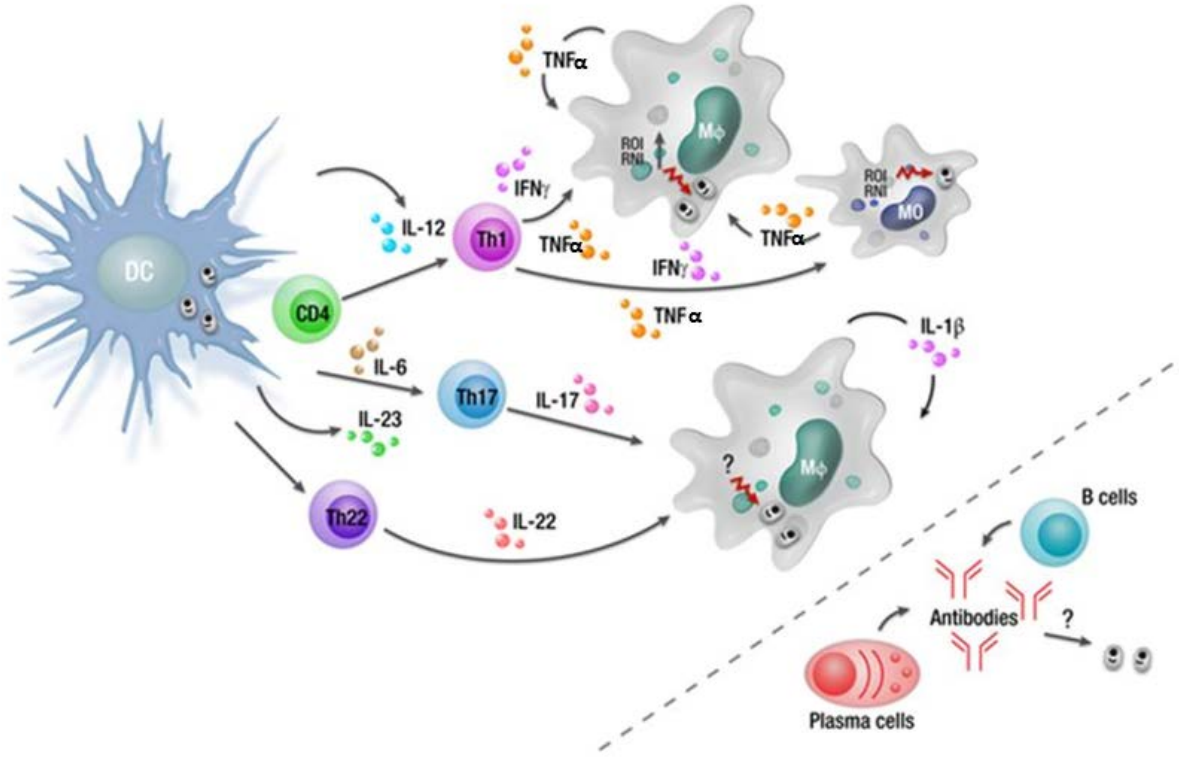

Figure 2.2 : Overview of cellular responses during an asymptomatic *L. donovani* infection.

Infected macrophages produce TNFα and IL-1β in response to *L. donovani* infection. However, DC IL-12 production in response to *L. donovani* infection is required to drive the differentiation of antigen-specific $CD4^+$ T cells into IFNγ- and TNFα-producing Th1 cells. These cells activate infected macrophages and monocytes to produce ROI and RNI that kill intracellular parasites. There are also reports in humans that Th17 and Th22 cells develop in asymptomatic, infected individuals, possibly driven by IL-23 and IL-6. However, the anti-parasitic mechanism mediated by these $CD4^+$ T cell subsets following *L. donovani* infection remains unknown. Although parasite-specific antibodies are readily detected in asymptomatic individuals, their role, if any, in control of infection and protection against reinfection is unknown. Abbreviations: MO, monocyte; Mφ, macrophage.

2.3.2 Development of chronic infection in the spleen and bone marrow

L. donovani infection in the spleen and bone marrow is characterised by parasite persistence and tissue damage [73, 93]. The marginal zone (MZ) and marginal metallophilic (MM) macrophages in the spleen [94] and stromal macrophages in the BM [93] are major

initial targets for infection. In the spleen, subsequent infection of red pulp (RP) macrophages by parasites also occurs [95]. Despite a small drop in parasite burden in the spleen 24 hours after infection, parasites numbers increase and then stabilise over the following 1-2 months, leading to a chronic infection [94]. A similar pattern of parasite growth also occurs in the BM [96].Chronic infection in the spleen leads to splenomegaly and structural changes in the macro-architecture of the tissue [97], such as disruptions to the MZ and loss of the follicular dendritic cells (FDCs), which correlates to the loss of the B cell follicles in the germinal centre (GC). These events may contribute to an inefficient immune response in the spleen during VL [73, 97].

The MZ of the spleen plays an important role in directing cellular traffic and is located between the macrophage beds of the red pulp and the T and B cells zones contained within the white pulp. The marginal zone macrophages (MZM), marginal metallophillic macrophages (MMM), sinus-lining reticular cells, marginal zone B cells and dendritic cells (DCs) make up the MZ [98]. During a chronic *L. donovani* infection, widespread remodelling of the MZ takes place, resulting in the loss of MZM and changes in cell surface molecule expression on MMM populations [97]. The loss of MZM during VL is associated with disrupted lymphocyte migration into the white pulp of the spleen [97]. Within the white pulp region, remodelling causes disruption to both the $gp38^{+}$ fibroblastic reticular cell (FRC) network, which guides T cell and DC migration to the T cell zone [99] and FDC network in the B cell follicles[73]. Studies by Dalton *et al* showed that by using a receptor tyrosine kinase inhibitor (RTKI) sunitinib maleate (Sm), vascular remodelling and splenomegaly associated with VL can be blocked and the effects can be reversed [100]. However, use of Sm alone did not cause a reduction in parasite burden in the spleen, but when used in combination with conventional antimonial drugs, enhanced leishmanicidal activity was

observed [100]. *L. donovani* infection also appears to promote development of regulatory DC's in the spleen. Examination of DC populations in the infected spleen showed an increase in $CD11c^{lo}$ $CD45RB^{+}$ DC's, compared to the $CD11c^{hi}$ DC population [101]. DC's with the $CD11c^{lo}$ $CD45RB^{+}$ phenotype secrete IL-10 when stimulated with lipopolysaccharide (LPS) and skew T cell development to IL-10 producing regulatory T (Tr1) cell responses [102] (Figure 2.3). The development of these regulatory DC's was promoted by stromal cells in infected spleens [101]. $CD11c^{lo}$ $CD45RB^{+}$ DC's show features of immature DC's, indicated by low expression of co-stimulatory molecules and intracellular MHC class II [101]. These DC's were capable of inhibiting mixed lymphocyte reactions (MLR's) driven by conventional DC's, and this effect could be reversed by the presence of an anti-IL-10 receptor monoclonal antibody (mAb) [101]. Comparative analysis of regulatory DC's ($CD11c^{lo}$ $CD45RB^{+}$) generated in the presence of naive spleen stromal cells and *L. donovani* - infected spleen stromal cells showed that the latter had elevated regulatory capacity, which could overcome the effects of anti-IL-10 receptor mAb. In addition to inhibiting MLR's, these DC's also had elevated levels of IL-10 mRNA accumulation, compared to $CD11c^{lo}$ $CD45RB^{+}$ DC's generated in the presence of naive spleen stromal cells [101].

Relatively few studies have been conducted to investigate the effect of *L. donovani* infection on the BM in experimental VL. However, work by Cotterell *et al.* showed that in BALB/c mice, *L. donovani* affects the regulation of haematopoiesis [93]. Stromal macrophages in the BM were found to be targeted by *L. donovani*, and following exposure to granulocyte macrophage colony-stimulating factor (GM-CSF) and TNFα, stromal macrophages were able to support increased level of myelopoiesis [93]. Related changes reported in VL patient BM include an increase in plasma cell numbers, erythroid hyperplasia and moderate to severe megaloblostosis [103].

The TNFα family of cytokines and their signalling molecules have an important role to play in the development of the splenic MZ [97]. Previous studies showed that during *L. donovani* infection, TNFα is expressed throughout the spleen and plays an important role in tissue remodelling in this organ. Studies using TNFα blockade, as well as studies in TNFα-deficient ($TNF\alpha^{-/-}$) mice infected with *L. donovani* showed that the loss of MZM was reduced, and although some structural changes in the spleen were observed, these were greatly reduced compared with control-infected animals [97]. One of the consequences of this overt TNFα production and subsequent impact on the MZ is thought to be that DC's and naive T cells fail to migrate to the periarteriolar lymphoid sheath (PALS) of the spleen, resulting in reduced priming of naive T cells [12].

Mice deficient in IL-10 fail to establish a substantial *L. donovani* infection, and blockade of IL-10 signalling during an established *L. donovani* infection dramatically enhances anti-parasitic immunity [13, 104, 105]. In addition, there is strong evidence that IL-10 plays a key role in regulating the expression of the programmed cell death (PD)-1 ligands (PD-L1 and PD-L2) on APCs [106], and there has been a report that the splenic environment during chronic VL is associated with the increased expression of PD-L1 on DC's [107]. Furthermore, following ligation of PD-L1 to its receptor PD-1 found on T cells, there is diminished T cell proliferation and cytokine production [108]. Blocking PD-L1 ligation during *L. donovani* infection results in increased $CD8^+$ T cell survival and partially restoration of the functional capacity of these cells [12]. The partial restoration of $CD8^+$ T cell functionality indicates that there may be several other important immune regulators that also suppress cytokine production by these cells.

IL-27 has been shown to play a major role in the induction of IL-10 producing T cells [109]. A study in mice revealed that IL-27 drives the expansion and differentiation of IL-10-producing Tr1 cells, promoting c-maf-mediated IL-21 production, which acts as an autocrine growth factor for the expansion and/or maintenance of IL-27-induced Tr1 cells [110] (Figure 2.3). IL-27 belongs to the IL-12 cytokine family, and previously, IL-27Rα-deficient mice infected with *Toxoplasma gondii* were found to develop a normal Th1 response, but then died when this response became severely dysregulated [111]. IL-27 has been reported to play critical roles experimental *Leishmania* infection. IL-27Rα-deficient mice infected with *L. donovani* developed an enhanced Th1 responses, but severe liver pathology was also observed in these mice [112]. In non-healing *L. major* infection, IL-27 was also found to regulate IL-10 and IL-17 production by $CD4^+$ cells [113]. Thus, IL-27 signalling appears to be important for the generation of IL-10 during experimental leishmaniasis, and one way this cytokine may regulate host immune responses might involve regulating expression of PD-1 and its ligands.

Although studies in the spleen and BM of *L. donovani* infected mice have provided a better understanding of the immune mechanisms associated with progressive and chronic infectious diseases, studies on disease models have limitations and ultimately discoveries need to be validated in humans if they are going to be used to improve disease treatments or design better vaccines.

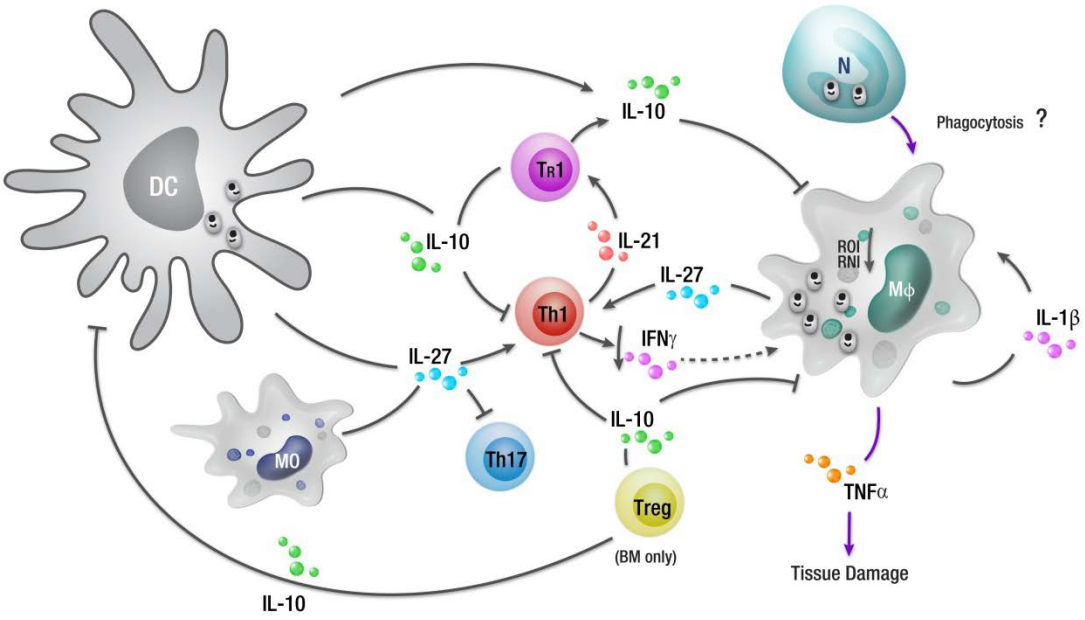

Figure 2.3 : Overview of cellular responses during a chronic *L. donovani* infection.

During an established *L. donovani* infection, a subset of regulatory DC's in the spleen can produce IL-10 that promotes the expansion of IL-10-producing regulatory T cells (Tr1), as well as inhibiting antimicrobial mechanisms in macrophages and other phagocytic cells (including suppression of ROI and RNI generation). IL-27 produced by regulatory DC's and macrophages, along with T cell–derived IL-21, can drive the differentiation of Th1 cells into Tr1 cells, as well as inhibit Th17 development. IL-10 produced by Tr1 cells can suppress antigen presentation, contributing to T cell dysfunction, as well as down-regulate $CD4^+$ T cell IFNγ production. There has been a report that IL-10 can also be produced by Treg cells in the BM of VL patients. Although uptake of infected neutrophils undergoing apoptosis by macrophages contributes to the establishment of *L. major* infection in mice, no such mechanism has yet been described during *L. donovani* infection. Abbreviations: N, neutrophil.

2.4 THE ROLE OF $CD4^+$ T CELLS DURING INFECTION

Studies in experimental VL have highlighted the importance of $CD4^+$ T cell responses in the liver during granuloma formation and control of parasite burden in a TNFα and IFNγ dependent manner [114]. Early experiments carried out by Stern *et al* show that $CD4^+$ T cells

are critical for development of resistance against *L. donovani* in BALB/c mice. This study showed that liver parasite burdens in anti-CD4 antibody treated mice were 2.6 fold higher compared to controls [65], indicating the need for $CD4^+$ T cells for parasite clearance. Histological analysis of livers of mice treated with anti-CD4 antibody showed that the granuloma formation was poorly defined, with highly infected KC surrounded by poorly formed mononuclear cell aggregates [65]. $CD8^+$ T cells are also required to generate an immune response during VL [65], although they appear to play a relatively minor role. Recent studies showed that $CD8^+$ T cells purified from *L. infantum* infected mice exhibited cytotoxic activity and expressed Th1 cytokines (IFNγ and TNFα) [115]. Another study suggested that *Leishmania* parasites escape cellular responses by inducing exhaustion in $CD8^+$ T cells [107]. Studies by Stager *et al*., suggested that priming of $CD8^+$ T cells via vaccination can induce protection against *L. donovani* infection [116].

2.4.1 The activation of $CD4^+$ T cells by DC's

Interactions between antigen bearing DC's and naive T cells are important for inducing immune responses following *L. donovani* infection [117]. Interleukin-12 (IL-12) is a key cytokine that plays an important role in innate and antigen-specific responses in VL [86, 118]. IL-12 activates T cells to generate IFNγ, required for the activation macrophages and other leishmanicidal responses [119].

Neutralising IL-12 activity during *L. donovani* infection in BALB/c mice resulted in delayed parasite resolution in the liver, disruption of granuloma formation and reduced IFNγ production [86]. In the spleen, no change was observed during the first 28 days p.i. However,

at later time points, neutralisation of IL-12 resulted in increased parasite load, indicating that IL-12 has no effect on early parasite replication in the spleen but is critical for the development of immune responses required for the control of parasite burden later in the spleen [86].

Studies carried out by Gorak *et al.*, showed that one day after *L. donovani* infection, clusters of IL-12 $p40^+$ cells were observed in the white pulp region of the spleen. Closer analysis of the IL-12 $p40^+$ cells identified them as DC's [94]. Further analysis suggested that $CD8^+$ DCs are the primary producers of IL-12p40, and that peak production occurred 5 hours post infection (p.i.) in the spleen [120].

2.4.2 The role of $CD4^+$ T cells in resolving *L. donovani* infection in the liver

As mentioned previously, $CD4^+$ T cells are important for the resolution of disease during *L. donovani* infection [65]. Hepatic granuloma formation is required for clearance of parasites in the liver and is T cell dependent [121]. Early studies by Stern *et al.*, suggested that $CD4^+$ T cells are the primary produces of IFNγ during an *L. donovani* infection. They observed that nude mice reconstituted with immune spleen cells were able to generate substantial IFNγ in response to mitogen and parasite antigen. Further analysis revealed that $CD4^+$ T cells were the major producers of IFNγ [65]. IFNγ is required for anti-leishmanicidal activity and is vital for macrophage activation [122]. Treatment of *L. donovani* infected BALB/c mice with anti-IFNγ mAb resulted in uncontrolled parasite growth and disruption of granuloma formation [122].

TNFα, along with IFNγ, is crucial for macrophage activation and parasite clearance [76, 123]. However, studies in TNFα-deficient C57BL/6 mice showed that TNFα was critical for control of parasite growth and also contributed to granuloma assemble in the liver [76]. Interestingly, mice lacking TNFα are the only known mouse lines in which an *L. donovani* infection is lethal.

IL-12, along with IFNγ and TNFα, also plays a positive role in controlling an *L. donovani* infection, while Interleukin 10 (IL-10) has suppressive effects on the immune function of T cells. In IL-$10^{-/-}$ mice, resolution of the *L. donovani* infection is achieved quickly, compared to wild-type control mice, and treatment with anti-IL-10 receptor mAb rapidly promotes clinical cure [104, 105].

Besides cytokines, chemokines are also important mediators of immune response against *L. donovani*. Infection with *L. donovani* brings about a rapid T cell-independent chemokine response followed by the amplification of this response, which is T cell-dependent [124]. Patients with VL show high levels of CXCL9 and CXCL10 in their serum, although the roles for these cytokines in controlling infection is not clear [125]. In the livers of *L. donovani* infected mice, high levels of MIP-1, CCL2 and CXCL10 are observed [124]. MIP-1 and CCL2 produced by infected KCs are involved in attracting monocytes and neutrophils to the liver. Resident $CD4^+$ and $CD8^+$ T cells in the liver promote and maintain levels of CXCL10, which provides the stimulus for granuloma formation. Infiltrating immune cells, along with T cells aggregate around the infected KCs to form the hepatic granuloma in the liver [124].

2.4.3 Organ-specific roles for $CD4^+$ T cells during VL

Studies carried out by Kenney *et al.*, have shown that in the spleen of VL patients, a mixed Th1/Th2 response is observed. Analysis of serum from these patients revealed detectable levels of IFNγ and IL-10. Treatment with exogenous IFNγ, to assist with parasite clearance, also resulted in increased levels of serum IL-10, suggesting an interdependent relationship between these cytokines during disease [126]. Early studies carried out by Sacks *et al.* showed that T cells taken from *L. donovani* infected patients were not responsive to the *Leishmania* antigen and deletion of $CD8^+$ T cells did not reverse the unresponsiveness to parasite antigen [21]. Although our understanding of the immune mechanisms required for resolution of infection in the liver in experimental VL is extensive, we know relatively little about why $CD4^+$ T cells do not control infection in humans or the spleen and bone marrow of susceptible mice.

2.5 POST KALA-AZAR DERMAL LEISHMANIASIS AND HIV CO-INFECTION

Post kala-azar dermal leishmaniasis (PKDL) is a complication of VL characterised by nodular, macular or a maculopapular rash on individuals who have recovered from VL [127]. PKDL appears in individuals after apparently successful VL treatment, possibly caused by suppression of immunity in the skin to persisting parasites [128, 129]. PDKL is mainly observed in the Indian subcontinent and East Africa, where an estimated 10-20% of cases in India and 50-60% of cases in the Sudan progress to PKDL after VL treatment [130]. Indian PKDL appears two to seven years, or even decades after the VL treatment, while in the Sudan it appears earlier (six to seven months after treatment) [130]. In some cases, there may be no

previous history of leishmaniasis [131, 132]. PKDL cases are of epidemiological importance because these patients can serve as parasite reservoirs [130]. PKDL is difficult to treat and drugs used include sodium antimony gluconate (SAG), amphotericin B and miltefosine, depending on geographical location and clinical setting. The long duration of treatment and high drug doses required for clinical effects increases chances of drug toxicity, as well as increasing the risk of parasites developing drug resistance [130, 133].

Immunological features of PKDL differ from VL in several ways [133]. In VL, a suppressed CMI response is observed, which is restored on successful treatment and most cured individuals are resistant to re-infection [134]. PDKL on the other hand arises in a proportion of cured VL patients, due to the suppression of immune response against parasites present in the skin [135, 136]. PKDL cases studied in Sudan show an increase in $CD3^+$ T cell infiltration within lesions containing *Leishmania* parasite or antigen, and IFNγ, IL-10 and IL-4 are the main cytokines produced in the inflamed lesions [137]. In another Sudanese study, Gasim *et al.*, showed that PKDL could be predicted by assessment of IL-10, as high levels of IL-10 were observed in plasma and keratinocytes of VL patients which developed PKDL, compared to VL patients that did not [138]. A subsequent study by the same group also reported a positive association between the onset of PKDL and an increase in circulating parasite-specific PBMC, evident by the stronger parasite-specific T cell responses [139]. In India, increased CMI responses were also observed in patients at the onset of PKDL, compared to during chronic PKDL [140]. However, another study failed to find detectable antigen-specific immune responses in Indian PKDL patients [141], while Ganguly *et al*., reported that CMI responses were present in Indian PKDL patients, but were dominated by antigen-specific IL-10 production by $CD8^+$ T cells [68]. Clearly, further studies are required to both identify predisposing immune factors associated with PKDL development, as well as

to better define dysfunctional immune pathways operating during this serious disease complication.

$CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells are a sub-population of $CD4^+$ T cells involved in immune homeostasis with the potential to produce IL-10 during inflammation [142]. Studies by Katara *et al*., showed that Treg cell markers and IL-10 were elevated in tissue samples from PKDL patients, compared to tissue taken from healthy controls [143]. Furthermore, Treg cells were found to aggregate in tissue lesions of patients with PKDL, where there was a positive association between parasite burden, certain Treg cell markers and IL-10 levels [143]. In another recent study, elevated IL-17, IL-23 and RORγt mRNA accumulation was found in PKDL lesions, compared with tissue after drug treatment, and this was accompanied by increased IL-17 and IL-23 plasma levels [144]. Thus, although PKDL is accompanied by IL-10-mediated immune suppression in many cases, the picture is not always clear, and may also involve other deregulated inflammatory responses. Again, this is an area requiring further investigation at the molecular and cellular level.

Recent studies have shown that secondary infections are also common in patients with VL, possibly due to the marked immune suppression observed in infected patients [13]. *L. donovani /L. infantum* co-infection with the Human Immunodeficiency Virus (HIV) has now been recognised as a significant clinical problem [145]. A higher mortality rate has been reported in AIDS patients co-infected with *L. donovani /L. infantum*, compared to other co-infections [146]. HIV patients with VL have enhanced pro-inflammatory cytokine responses, associated with increased HIV viral load, which can accelerate the progression from asymptomatic HIV to AIDS [147, 148]. In addition, parasite multiplication promotes the

survival, proliferation and elevated levels of cellular dNTP in human monocytes which can also accelerate HIV replication [149, 150]. Treatment of VL in HIV patients involves the use of standard drugs, but due to the enhanced immune suppression in HIV patients and the partial reliance on host immune mechanisms for drug efficacy [128], these treatment strategies are often inadequate, and in most cases, patients are unresponsive to drug treatment [128]. Furthermore, those patients who do respond to treatment often relapse, possibly due to the low $CD4^+$ T cells numbers associated with HIV infection and parasites persisting after drug treatment [151]. Host T cells responses are abrogated in HIV infection, including skewing away from Th1 responses [152] which are required for effective leishmanicidal responses [65]. Wolday *et al.* showed that PBMCs from *Leishmania* /HIV co-infected individuals produce low levels of IL-12 and IFNγ, and higher levels of IL-4 and IL-10 following stimulation with parasite antigen [153]. IL-15 is involved in promoting and maintaining Th1 responses, and was also decreased in plasma from patients with *Leishmania* /HIV co-infection [154]. Hence, as might be predicted, these results suggest that HIV infection suppresses anti-parasitic Th1 immune response required for parasite clearance [151].

2.6 **PAST AND CURRENT TREATMENT OPTIONS**

Prompt detection and treatment of VL is critical to achieve cure and prevent transmission of disease. Left untreated, VL results in increased rates of morbidity and mortality in endemic regions of the world [155].

2.6.1 Current anti-leshmania drugs available for the treatment of VL

The choice of treatments against VL is largely based on the region where the infection was acquired, the local responses to treatment and the treatment resistance pattern [156]. Pentavalent antimonials, such as sodium stibogluconate, pentostam, meglumine antimonite and glucantime, have been the mainstay of antimonial therapy for the last 60 years [157]. However, there is now considerable parasite resistance against these drugs, especially in North-Eastern India and surrounding areas [3]. Therefore, although these drugs are still employed to treat VL in Africa, drugs such as Amphotericin B, Miltefosine, aminosidine (paromomycin) and sitamaquine have been developed as effective treatments against VL in areas of antimonial drug resistance [3]. However, these drugs are still far from ideal because of cost, toxicity, development of parasite drug resistance after prolonged use and the duration of treatment [4]. Although some progress has been made recently in addressing this latter issue [158], where a single dose of lipid formulation of Amphotericin B (Ambisome), was effective in treating VL patients with lower toxicity outcomes compared to the conventional drug treatment. Table 1 list the current anti-leishmania drugs available to treat VL.

Combination drug therapy involving administration of two anti-leishmania drugs is a new therapeutic approach which is being tested as a way to increase efficacy, reduce development of parasite resistance and reduce treatment duration and cost [159]. A recent study by Sunder *et al.* in a cohort of 613 patients, indicated that combination of various antimonals were more effective and less toxic than treatment with amphotericin B alone [158]. Other interesting approaches currently being studied are targeted therapy and immunotherapy. Targeted therapies involves blocking of essential biochemical or signalling

pathways that are required for *Leishmania* parasite growth and survival, while, immunotherapy encompasses the use of biological molecules or compounds such as cytokine therapy [160], alone or in combination with anti-leishmania drugs to modify immune responses [161].

Each VL case signifies our failure to prevent leishmaniasis, and each death our failure to treat the disease soon enough. Until disease eradication has been achieved, chemotherapy will remain critical to prevent morbidity and mortality caused by VL. There is an urgent need for the development of alternative and effective therapies against VL. The development of compounds or agents that selectively inhibit key cellular pathways that promote *Leishmania* parasite growth and maintenance has opened a new avenue for anti-leishmania therapy. The use of targeted therapy and immunotherapy could address current challenges associated with treatment and management of VL by reducing the cost of therapy, development of drug resistance to currently available chemotherapies and decreasing the dose or length of treatment. Therefore, a major component of this thesis is dedicated to investigating the effect of targeted immunotherapy on the outcome of experimental VL.

Drugs	Dosage and route of administration	Mode of action	Efficacy of drug (%)	Advantages	Limitations	References
Amphotericin B	0.75-1.0 mg/kg; 15-20 i.v. infusions given daily or every alternate day	Forms complexes with the parasite by binding to membrane ergosterol resulting in increased membrane permeability, which alters the ion balance, resulting in parasite death.	$\geq$ 95%	Effective in antimony drug resistant regions	Costly, toxic and requires longer the duration of treatment	[162, 163]
Liposomal Amphotericin B (Ambisome)	10 mg/kg i.v.as a single dose OR 3 mg/kg/day i.v. for 5 days	Mechanism of action is similar to Amphotericin B, although delivery of the drug is directly to infected macrophages.	$\geq$ 96%	Extremely effective; Shorter treatment duration; No documented resistance	Costly	[164]
Miltefosine	50-100 mg/day for adults depending on weight. Oral drug.	Interacts with the surface membrane of *Leishmania* parasite. Parasite death is mediated by apoptosis	85-95%	It's an oral drug. Primary treatment in the Indian subcontinent	Costly and toxic	[163, 165]
Paromomycin (a.k.a. animosidine)	11-15 mg/kg daily intramuscularly (i.m.) for 21 days	Induces reduction in parasite mitochondrial membrane potential and inhibits protein synthesis	95%	Acts co-actively with other antimonials and is a low-cost drug for VL	Toxic and in-effect in East Africa	[166-168]
Pentavalent antimonials	20 mg/kg/day (i.m.) for 20-30 days	The drug is converted into the active trivalent form in the amastigote/ macrophage vacuole and exposes the parasite to oxidative stress of the host cell	80-90%	Low-cost drug	Toxic and increased drug resistance	[169, 170]

Table 1: Current anti-leishmanial drugs against VL.

Adapted from a review by S. Sundar and colleagues [161].

2.6.2 Vaccines against VL

At present there is no effective VL vaccine for humans. The development of a vaccine to prevent leishmaniasis has been a long-term goal for researchers in the field. One of the major hurdles for developing vaccines to either prevent or treat VL, has been a limited understanding of the precise immune mechanisms required for controlling parasite growth without causing disease. Because of the intrusive techniques required to analyse tissue in VL patients, our current understanding of the host immune response during VL is largely derived from studies performed in *L. donovani*-infected, genetically susceptible mice.

Past studies suggest the use of whole parasite vaccines as an approach to control VL. The process of "leishmanisation" involves the deliberate infection of people with cutaneous leishmaniais (CL) causing parasite species on unexposed areas of the body to establish an infection that is controlled in most individuals, resulting in long-term protection [6]. This technique was practised for centuries throughout the Middle East and parts of Asia, and large-scale trials were carried out in the former Soviet Union and Israel with some success [171, 172], as long as the parasites used were viable and infective [173]. However, despite the solid immunity that develops in most individuals, this approach has largely been abandoned as some individuals developed complications such as large skin lesions, exacerbation of skin diseases and poor responses to vaccines [7, 8]. To avoid this, strategies have been shifted to attenuated or killed whole parasite vaccines. These types of vaccines are non-pathogenic and non-replicative but have the ability to generate anti-parasitic immune responses in immunised hosts.

Studies have already shown that γ-irradiated whole *Leishmania* parasite can be used as a vaccine against experimental CL [174]. In a recent study carried out by Datta *et al.*, prophylactic and protective immune responses were generated in mice immunised intramuscularly (i.m.) with radio-attenuated *L. donovani* parasites [175, 176]. They showed that in immunised mice, there was a higher Th1 cytokine response and suppressed Th2 responses, associated with lower parasite burdens, relative to control animals. These results were indicative that whole attenuated parasites can be used as effective vaccines.

For the effective control of VL in endemic areas, a vaccine is deemed critical. However, vaccine development faces several challenges either due to host evasive mechanisms of parasites or heterogeneity in the human population resulting in poor immune responses following vaccination. A key step in vaccine design is the identification of suitable vaccine candidates, which stimulates a host protective immune response. Hence, another component of this thesis is dedicated to investigating the generation of a whole parasite vaccine to protect against experimental VL using different forms of attenuation in the presence or absence of adjuvants.

Chapter 3: Materials and Methods

3.1 MICE AND PARASITES

3.1.1 Mice

Female C57BL/6 were purchased from the Animal Resources Centre (Canning Vale, Western Australia) and the Walter and Eliza Hall Institute for Medical Research (Victoria, Australia), and maintained under conventional conditions at the QIMR Berghofer Medical Research Institute (QIMR Berghofer). B6.FoxP3-GFP-DTR [177], B6.NKp46Cre.iDTR [178] and B6.RAG1$^{-/-}$ mice [179] were bred at the QIMR Berghofer. Unless otherwise stated, 6- to 10-week-old males and female, sex- and age-matched mice were used for all experiments. All animal procedures were approved by the QIMR Berghofer Animal Ethics Committee. This work was conducted under QUT Ethics Approval Number 1200000384 and QIMR Berghofer animal ethics approval number A02-634M, in accordance with the "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" (Australian National Health and Medical Research Council).

3.1.2 Parasites

L. donovani (LV9) parasites were maintained by passage in B6.RAG 1$^{-/-}$ mice. Briefly, B6.RAG 1$^{-/-}$ mice were infected intravenously (i.v.) via the lateral tail vein with 2x10^{7} amastigotes in 200 µL RPMI 1640 (QIMR Berghofer). Following the establishment of

chronic infection (approximately two months post infection), mice were killed by CO_2 asphyxiation and amastigotes were isolated from the spleens (see 3.1.3) to set-up infections.

3.1.3 Isolation of parasite for infection of mice

Amastigotes were harvested from infected B6.RAG $1^{-/-}$ mice spleens. The isolated spleens were homogenised to a single cell suspension in RPMI 1640, supplemented with 100 µg/mL streptomycin (Invitrogen, VIC, Australia) and 100 U/mL penicillin (Invitrogen) (RPMI/PS) and centrifuged at 128 x g (Eppendorf Centrifuge 5810R) at room temperature (RT) for 5 minutes to remove cellular debris. The retained supernatant was centrifuged at 1800 x g at RT for 15 minutes and the pellet retained. Red blood cells (RBCs) were lysed with RBC Lysing Buffer (Sigma-Aldrich, Castle Hill, Australia), as per the manufacturer's instructions. Amastigotes were pelleted by centrifugation at 1800 x g at RT for 15 minutes. The supernatant was discarded and the pellet washed two more times by resuspending in RPMI/PS and centrifuging at 1800 x g at RT for 15 minutes. Amastigotes were finally resuspended in 10 mL of RPMI/PS. The amastigote suspension was passed through a blunt 26-gauge needle (Terumo Corporation, Tokyo, Japan) 10 times, to ensure lysis of all host cells and to reduce clumping of parasites. Amastigotes were counted using the Helber bacteria counting chamber (Weber Scientific International Ltd, Teddington, UK). Mice were infected by injecting $2x10^7$ amastigotes i.v. via the lateral tail vein. In experiments examining low dose infections, mice were infected with $5x10^6$ amastigotes i.v.

3.1.4 *In-vitro* culturing of *L. donovani* promastigotes

L. donovani amastigotes were isolated as described (in section 3.1.3). The amastigotes were cultured in promastigote growth medium (cM199) consisting of M199 (Sigma-Aldrich) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS) (Life Technologies Australia Pty Ltd, VIC, Australia), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 40 mM HEPES (QIMR Berghofer), 0.1 mM adenosine (Sigma-Aldrich) (in 50 mM HEPES), 5 mg/mL hemin (Sigma-Aldrich) (in 50% triethanolamine (Sigma-Aldrich)), and 1 mg/mL 6-biotin (Sigma-Aldrich) for 21 day at 27°C in a 5% (v/v) CO_2 incubator, with media topped up 10 days after initiating cultures. 21 days post-culture, promastigotes were pelleted by centrifugation at 1800 x g at RT for 15 minutes. The supernatant was discarded and the pellet washed two more times by resuspending in RPMI 1640 and centrifuging at 1800 x g at RT for 15 minutes. Promastigotes were finally resuspended in 10 mL of RPMI 1640 and passed through a blunt 26-gauge needle 10 times to reduce clumping of parasites. Promastigotes were counted using the Helber bacteria counting chamber as described for amastigotes above. Parasites were either irradiated or chemically-attenuated (see section 3.1.5 and 3.1.6) and injected into mice.

3.1.5 Irradiation of *L. donovani* parasites

Irradiation of *L. donovani* (LV9) parasites (amastigotes or cultured promatigotes) was carried out by gamma irradiation (Gammacell® 40 Exactor Low Dose-Rate Research Irradiator (Best Theratronics) with a Cesium-137 source (1.1 Gy/minute) housed at QIMR Berghofer). LV9 parasites were irradiated at 500 (10 hrs), 1000 (20 hrs) and 1500 (30 hrs)

Gy, after which parasites were washed twice in RPMI/PS, counted, and the immunizing dose calculated. Mice were immunized i.v. or intraperitoneally (i.p.) with the required number of parasite (as described in Chapter 6).

3.1.6 Chemical-attenuation of *L. donovani* parasites

Chemical-attenuation of *L. donovani* (LV9) parasites (amastigotes or cultured promatigotes) was carried out using TH-III-149 (tafuramycin A: TFA) (kindly provided by Dr. Michael Good), as previously described [180]. TFA was dissolved in DMSO (Sigma-Aldrich) at 10,000× the desired attenuating concentration. Stock solutions of PET (polyethylene glycol 400, absolute ethanol, and Tween 80 in 6:3:1 proportions) (Sigma-Aldrich) and 5% (w/v) glucose (Sigma-Aldrich) were made and mixed 1:2. The TFA/DMSO solution was then added at 10% volume to the PET/glucose mix. TFA was diluted to a 20 μM final concentration in RPMI 1640 and then *L. donovani* parasites were added in RPMI 1640 to give a final concentration of 2 μM. The mixture was then incubated at 37°C in a 5% (v/v) CO_2 incubator for 40 minutes with frequent agitation/rocking. The parasites were washed and resuspended in RPMI 1640 and incubated at 37°C in a 5% (v/v) CO_2 incubator for 20 minutes. The parasites were washed once with RPMI 1640, counted and the immunizing dose was calculated. Mice were immunized i.v. with the required number of parasite (as described in Chapter 6).

3.1.7 Adjuvants used for immunization

The adjuvants CpG ODN sequence 1826 (TCCATGACGTTCCTGACGTT) and control sequence 1982 (TCCAGGACTTCTCTCAGGTT) were purchased from Sigma-Aldrich. Poly I:C was purchased from Integrated Sciences Ltd. (NSW, Australia). Mice were injected i.v. with 50 μg CpG ODN 1826, 50 μg CpG ODN 1982 or 50 μg Poly I:C (as described in Chapter 6).

The immunization regime and parasite number used are described in Chapter 6. Age matched control (AMC) mice were infected with $2x10^7$ amastigotes i.v. on the day indicated and housed in similar conditions as the immunized mice.

3.2 SAMPLE COLLECTION

At the times indicated in the text, mice were sacrificed by CO_2 asphyxiation. Body and organ weights were recorded for each animal in grams (g).

3.2.1 Collection of blood for serum isolation

Mice were bled via cardiac puncture and the blood was collected in Minicollect Lithium heparin tubes (Greiner Bio-One, Austria). Blood serum was obtained by centrifuging the Minicollect tubes at 775 x g (Eppendorf Centrifuge 5418) at RT for 10 minutes and the

upper layer of serum was transferred into a 96 well round bottom plate (Sigma-Aldrich) and stored at -20^{o}C until required.

3.2.2 Collection of animal organs

After mice were bled, the spleen and livers were removed from each animal and weights were recorded in grams (g). Spleens removed from the animals were collected in 10 mL of RPMI/PS. Livers were perfused via the hepatic portal vein using 10 ml perfusion buffer (1X PBS (QIMR Berghofer)). Perfused livers were collected in 5 mL of 2% (v/v) FBS-supplemented PBS.

3.2.3 Assessment of parasite burdens

Parasite burdens were determined from impression smears of spleens and livers. Briefly, a section of the spleen and hepatic lobes were taken and blotted on filter paper (Whatman, UK) to remove excess blood and then pressed onto a glass microscope slide (Universal Choice Wholesaler, NSW, Australia) to produce multiple impression smears. The smears were air dried and stained with Diff-Quik (Lab Aids, Narrabeen, Australia), according to manufacturer's instructions. Parasite burden was expressed as Leishman Donovan Units (LDU), calculated by multiplying the number of parasites per 1,000 host nuclei by the organ weight in grams [73].

LDU= no. of parasites per 1000 nuclei x organ weight (g)

Spleen parasite burden was also determined by limiting-dilution analysis [181]. Briefly, Bacto beef (5 g; BD Biosciences, NSW, Australia), Neopeptone (2 g; BD Biosciences) Agar (2 g; BD Biosciences) and NaCl (0.5 g; Sigma-Aldrich) were dissolved in 100 mL of double-distilled H_2O and autoclaved for 30 minutes. Agar heated to 56°C was dispensed into a flat-bottom, 96-well plate (50 µl per well) (Sigma-Aldrich). Agar plates were allowed to set at a 45° angle and were stored at 4°C before use. Three hours before the addition of spleen mononuclear cells (MNCs), the plates were placed at RT. Splenic mononuclear cells (MNCs) were isolated, as described in Section 3.3.2. Single-cell suspensions were diluted 1/10 in RPMI 1640 media, supplemented with 20% (v/v) heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 40 mM HEPES and 10 µM Hemin (in 50% triethanolamine). Serial dilutions of cell suspensions were made, and 100 µL was added to each of 16 replicate wells. The plates were cultured in a humidified, 5% (v/v) CO_2 incubator at 25°C to 26°C for 9 days. The number of wells negative for parasite growth was then scored using an inverted microscope. The logarithm of the fraction of negative wells was plotted against the number of host cells plated at each serial dilution. The equation of the best-fit line was generated by a χ^2 minimization method, using Statistical Analysis System (SAS) software. The cell dilution yielding a fraction of negative wells for parasite growth gives an estimate of the reciprocal parasite frequency.

3.3 CELL ISOLATION AND PREPARATION

3.3.1 Hepatic Mononuclear Cell (MNC) preparation

Perfused livers collected in 5 mL of 2% (v/v) FBS-supplemented PBS (wash buffer) were passed through a 200 µM metal mesh (Sefar Pty Ltd, QLD, Australia), placed in a tea sieve. Cell suspension was washed twice in wash buffer by centrifugation at 338 x g for 6 minutes at RT. The cell pellet was resuspended in a 33% (v/v) Percoll (GE Healthcare Australia Pty. Ltd., NSW, Australia) density gradient and centrifuged at 578 x g for 15 minutes (no brake). The cell pellet obtained was suspended in RBC lysing buffer and RBC removed, as per manufacturer's instructions. Cells were washed twice and resuspended in 5 mL RPMI/PS. Cells were counted using a haemocytometer.

3.3.2 Splenic MNC preparation

Collected spleens were passed through 100 µM mesh sieve (Corning, USA) (a single sieve for each spleen). Cells were washed once in RPMI/PS by centrifugation at 338 x g for 6 minutes at 4^{o}C. RBCs were lysed using RBC lysing buffer, according to the manufacturer's instructions. Cells were washed once and resuspended in 10 mL RPMI/PS. Cells were counted using a haemocytometer.

3.3.3 Isolation of peritoneal macrophages

Peritoneal macrophages were collected by conducting a peritoneal lavage. Briefly, mice were euthanized by CO_2 asphyxiation. Using scissors and forceps the outer skin of the peritoneum was cut and pulled back to expose the peritoneum lining the peritoneal cavity. 5 mL of ice cold D-PBS (1X) (Sigma-Aldrich) was injected into the peritoneal cavity using 27g needle. After injection, the peritoneum was massaged. The fluid containing peritoneal cells was then collected using a 5 mL syringe and 25g needle. Cells collected were washed with cold D-PBS and resuspended in cold, complete DMEM (cDMEM), consisting of DMEM (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated FBS, 10 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were counted using a haemocytometer.

3.3.4 Magnetic cell sorting (MACS) purification of DC's

Mouse spleens were processed to obtain a single-cell suspension as mentioned previously (see 3.3.2). $CD11c^+$ DC were positively selected from single-cell suspensions using magnetic-activated cell sorting (MACS). 10^8 cells from each sample were incubated in 400 µL MACS buffer (PBS, 1% BSA (Sigma-Aldrich), 5mM EDTA (Sigma-Aldrich)) with 100 µL metallo-conjugated anti-mouse anti-CD11c beads (N418) (Miltenyi Biotech, Germany) for 15 minutes in the refrigerator (4-8°C). Cells were washed by adding 1-2 mL MACS buffer per 10^7 cells and centrifuged at 338 x g for 7 minutes at 4^oC. Supernatant was discarded and cells were resuspended in ice-cold MACS buffer and passed through a 50 µM sieve placed on a pre-wet (0.5 mL MACS buffer) MS MACS column (Miltenyi Biotec)

attached to a magnetic stand. The column was washed three times with 0.5 mL ice-cold MACS buffer, removed from the magnet and positively stained cells were flushed from the column into a collection tube using 1 mL of MACS buffer. The volume was made up to 10 mL with RPMI/PS and cells washed. Enriched DCs (30 - 40% purity checked by FACS) were resuspended in complete RPMI (5% cRPMI), consisting of RPMI 1640 supplemented with 5% (v/v) heat-inactivated FBS, 10 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were counted using a haemocytometer.

3.4 ANTIBODIES AND DRUGS FOR *IN-VIVO* ADMINISTRATION

3.4.1 *In-vitro* culturing of monoclonal antibodies

All hybridomas (anti-GITR [DTA-1], anti-IL-10R [1B1.3a], anti-IL-2 [S4B6 and JES6-1A12] and anti-NK1.1 [PK136] mAb) were grown in-house at 37˚C in a 5% (v/v) CO_2 incubator. Cell culture media consisted of RPMI 1640 supplemented with 5% (v/v) heat-inactivated FBS, 200 μM HEPES, 2mM sodium pyruvate (Sigma-Aldrich), 100 μg/mL streptomycin and 100 U/mL penicillin. Purified mAbs were prepared from culture supernatants by protein G column purification (GE Healthcare Australia Pty. Ltd.), followed by endotoxin removal (Mustang membranes, East Hills, NY). The concentration of purified mAbs was then measured by spectrophotometry, resuspended at 2 mg/mL in 0.9% sodium chloride (Baxter, NSW, Australia) and stored at -20˚C.

Anti-CD4 (GK 1.5), anti-CD8 beta (β) (53-5.8), anti-CTLA-4 (UC10-4F10-11) and control Rat IgG mAb were purchased from BioXCell, (West Lebanon, NH, USA).

3.4.2 Intra-peritoneal administration of antibodies

All antibodies were administered i.p. by holding the mouse at a 45^{o} angle to avoid injuring vital organs in the peritoneal cavity. A 27-gauge insulin syringe (Terumo Corporation) was used. For *in vivo* stimulation of GITR, mice were injected i.p. with 0.5 mg anti-GITR mAb in 200 µl 0.9% sodium chloride per mouse on day 14 or day 28 post-infection. Anti-IL-10R and anti-CTLA-4 mAbs were administered initially in 0.1mg doses and later in 0.5 mg doses, via i.p. injection in 200 µl 0.9% sodium chloride per mouse on days 28, 30 and 33 post infection or days 14, 19 and 24 post-infection. Anti-CD4 and anti-CD8β mAb was administered in 0.5 mg doses, via i.p. injection in 200 µl 0.9% sodium chloride per mouse two days prior to infection/challenge (see chapter 6), and every three days post infection/challenge until the end of the experiment. Control mice were administered the same quantities of control rat IgG (0.5 mg) mAb at the same time points as the stimulatory, depleting or blocking mAbs.

3.4.3 Preparation and administration of IL-2/anti–IL-2 complexes

One and a half µg of recombinant murine IL-2 (eBioscience, San Diego, CA) was incubated with 50 µg of either S4B6 or JES6-1A12 mAb in saline, for 30 minutes at 37°C

prior to i.p. administration (see 3.4.2) into each mouse in a volume of 200 µL on days 14 and 21 post infection.

3.4.4 Preparation and administration of pentavalent antimonial drug

The pentavalent antimonial, sodium stibogluconate (Sb^v; Albert David Ltd, Kolkata, India), was administered i.p. at 500 mg/kg/day doses in 200 µL 0.9% sodium chloride per mouse on days 14 and 21 post-infection. In multiple-dosing experiments, mice were injected i.p. on days 14, 16, 18, 20, 22, 24 and 26 post-infection with Sb^v at 50 mg/kg/day doses in 200 µL 0.9% sodium chloride per mouse. The dose of Sb^v used was based on drug dosing used by others [182].

3.4.5 Preparation and *in- vivo* administration of Diphtheria toxin

Diphtheria toxin (DT) was purchased from Sigma-Aldrich, diluted in saline and stored at 4°C until required. Treg cells and NK cells were depleted in B6.FoxP3-GFP-DTR and B6.NKp46Cre.iDTR mice, respectively, by i.p. injection of DT at 8 ng/g body weight in 200 µl 0.9% sodium chloride, two days prior to infection and every three days post infection until the end of the experiment.

3.5 EXPERIMENTAL METHODS

3.5.1 *In-vitro* infection of Macrophages

Isolated peritoneal macrophages (see 3.3.3.) were seeded ($5x10^5$ cells) in 16-well, glass chamber slides (Lab-Tek, Rochester, NY) and incubated at 37°C in a 5% (v/v) CO_2 incubator for 24 hours. After 24 hours, non-adherent cells were removed and adherent cells washed with DMEM and infected with LV9 amastigotes at a multiplicity of infection (MOI) of 10:1 (in 200 µL DMEM). After incubation at 37°C in a 5% (v/v) CO_2 incubator for one hour, free amastigotes were washed off with RT DMEM and cells were cultured for another 24 hours at 37°C in a 5% (v/v) CO_2 incubator, in the presence or absence of exogenous IFNγ (10 ng/mL) (Bio-Scientific Pty. Ltd., NSW, Australia). On the following day, 50 µL of the culture supernatant was collected for measurement of nitrite levels using the Griess assay and 100 µL for cytokine measurement. Plates were washed twice with pre-warmed (at 37^oC in a water bath) D-PBS. Glass chambers were stripped and slides were fixed and stained using DIFF-QUICK. Percentage infectivity was calculated as number of parasites per 100 host macrophages.

3.5.2 Detection of nitrite using Griess assay

The Griess method was used to assess nitrite (NO_2^-) production in the supernatant of cultured infected macrophages (see 3.5.1). The NO_2^- levels were estimated by reducing the nitrate to nitrite with nitrate reductase and measuring the nitrite concentration

calorimetrically. Briefly, Griess reagent (2% Sulphanilamide and 0.2% Napthylethylenediamide in 5% Phosphoric acid) (Sigma-Aldrich) was added to cell supernatant (1:1 with sample) in a 96-well plate, along with the medium blank, and absorbance was measured at 540 nm following incubation for 25 minutes at RT. The amount of endogenous nitrite accumulated was calculated from a standard curve constructed with different concentrations of sodium nitrite.

3.5.3 DC activation assay

Isolated DCs (see 3.3.4) were seeded ($5x10^4$ cells) in 96 well round-bottom plates and cultured with chemically-attenuated LV9 promastigotes at a MOI of 1:10 (in 200 μL cRPMI) in the presence or absence of adjuvants CpG ODN 1826 and control CpG ODN 1982 (3 μg/mL) or Poly (I:C) (50 μg/mL). Plates were incubated at 37°C in a 5% (v/v) CO_2 incubator for 24 hours. On the following day 100 μL of culture supernatant was collected for measurement of cytokine levels.

3.5.4 Antigen-specific cellular analysis

Mouse splenic MNCs (see 3.3.2.) were diluted to $2x10^6$ cells/mL in 5% cRPMI and aliquoted in 96 well plates at $1x10^5$ cells/well. Cells were stimulated with fixed *L. donovani* amastigotes at $2x10^6$ parasites/well. Briefly, fixed amastigotes were prepared by incubating the parasites in 4% (w/v) paraformaldehyde (PFA; MP Biomedicals, CA, USA) for 30 minutes and washed twice with RPMI/PS. These paraformaldehyde-treated parasites were

then resuspended in cRPMI at $4x10^7$ parasites/mL before culturing. Cells were cultured in the presence of parasite antigen (triplicate wells were cultured; total volume in each well was 200 µL) for a period of 24 and 72 hours at 37^oC in a 5% (v/v) CO_2 incubator. Intracellular cytokine staining was performed on cultured cells at these time points and culture supernatants were harvested for measurement of cytokine levels.

3.5.5 Fluorescence activated cell sorting (FACS) analysis of cell surface markers

Hepatic and splenic MNCs (100 µL from cell suspensions) were added to 96 well plates and centrifuged at 338 x g for 3 minutes at 4^oC (wash) once in cold FACS buffer (PBS, 0.02% (v/v) FBS). Pelleted cells were resuspended in blocking buffer (TruStain fcX™ antibody, BioLegend, CA, USA) and incubated for 15 minutes on ice. Cells were washed as above. Dead cells were excluded from the analysis, by resuspending the pellet in 50 µL LIVE/DEAD Fixable Aqua Stain (Invitrogen-Molecular Probes, CA, USA), and incubating at RT for 15 minutes (protected from light). Cells were washed twice as above and the pellet was resuspended in 50 µL of the antibody master mix (antibodies diluted in cold FACS buffer) (Table 3.1 List of antibodies used for fluorescence-activated cell sorting (FACS)) and incubated for 30 mins on ice (protected from light). The plate was then washed and cell pellets were fixed in 1% PFA and stored at 4°C prior to acquisition on the LSRFortessa (BD Biosciences). Data were analysed with FlowJo software (TreeStar, OR, USA).

3.5.6 FACS analysis of intracellular cytokines and transcription factors

Ex-vivo cytokine production was measured by stimulating hepatic and splenic MNCs (100 µL from cell suspensions in 96 well plates) in 10% RPMI/PS supplemented with 10% (v/v) heat-inactivated FBS, with phorbol 12-myristate 13-acetate (PMA, 25 ng/mL) (Sigma-Aldrich) and Ionomycin (500 ng/mL) (Sigma-Aldrich) in the presence of Brefeldin A (10 mg/mL) (Sigma-Aldrich) for 3 hours at 37˚C in a 5% (v/v) CO_2 incubator. The cells were pelleted and supernatant discarded. Prior to intracellular and transcription factor staining MNCs were stained for cell surface markers, as previously described (see 3.5.5). Cells were then permeabilized using the Transcription Factor Staining Buffer Set (eBioscience, CA, USA), used according to the manufacturer's instructions. Briefly, fixation/permeabilization working solution was made by diluting fixation/permeabilization concentrate (1 part) with fixation/permeabilization diluent (3 parts). Working solution (100 µL) was added to each sample and left to incubate for 30 mins on ice (protected from light). Cells were washed twice with 200 µL permeabilization buffer (1X working solution of permeabilization buffer by diluting the 10X concentrate with distilled water) and the pellet was resuspended in 50 µL of the antibody master mix (intracellular and transcription factor antibodies diluted in cold permeabilization buffer) and incubated for 45 - 60 mins on ice (protected from light). The plate was then washed again and cell pellets were fixed in 1% PFA and stored at 4°C prior to acquisition on the LSRFortessa. Data were analysed with FlowJo software.

Antigen	Identifies	Conjugate	Clone Name	Manufacturer
CD11a	Activated $CD4^+$ T cells	FITC	M1714	Biolegend
CD122	IL-2Rβ	PE	5H4	Biolegend
CD132	IL-2Rγc	Biotin	TUGm2	BD Biosciences
CD25	Tregs	PE-Cy 7	PC61	Biolegend
CD4	$CD4^+$ T cells	BV 605	GK1.5	BD Horizon
		APC	GK1.5	Biolegend
		PerCP-Cy 5.5	RM4-5	Biolegend
CD49d	Activated $CD4^+$ T cells	PE-Cy 7	R1-2	Biolegend
		Biotin	R1-2	Biolegend
CD8α	$CD8^+$ T cells	AF 700	53-6.7	Biolegend
Foxp3	Tregs	FITC	150D	Biolegend
		APC	MF14	Biolegend
IFNγ	Protein	PE	XMG1.2	Biolegend
		APC	XMG1.2	Biolegend
		BV 421	XMG1.2	Biolegend
IL-10	Protein	PE	JES5-16E3	Biolegend
		APC	JES5-16E3	Biolegend
KLRG-1	Terminally differentiated cells	PerCP-Cy 5.5	2F1	eBioscience
NK1.1	NK cells	BV 605	PK136	BD Horizon
		APC	PK136	Biolegend
T-Bet	Th1 cells	APC	eBio4B10	eBioscience
		PE-Cy 7	eBio4B10	eBioscience
TCRβ	T cells	BV 421	H57-597	Biolegend
		PerCP-Cy 5.5	H57-597	Biolegend
TNFα	Protein	PE	MP6-XT22	Biolegend

Table 2 : A summary of the fluorophore-conjugated antibodies used for surface and intracellular staining

3.5.7 Measurement of cytokines in serum and cell culture supernatants

Serum and tissue culture supernatants were assessed for the presence of soluble cytokines using the Cytometric Bead Array Flex Sets (CBA) (BD Biosciences) system and the HTS system plate reader on the LSRFortessa, as per manufactures instructions. Cytokine quantification was determined using FCAP Array Version 3 software for Windows (BD Biosciences).

3.6 **STATISTICAL ANALYSIS**

Comparisons between two groups were performed using non-parametric Mann-Whitney tests in mouse studies. Comparisons between multiple groups were made using a Kruskal-Wallis test and corrected using Dunn's multiple comparisons test. Differences of $p< 0.05$ was considered significant ($p<0.05$ = *; $p<0.01$=**; $p<0.001$=***; $P<0.0001$=****). Graphs depict mean values ± SEM. All statistical analyses were performed using GraphPad Prism 6 software for Windows (GraphPad, CA, USA).

Chapter 4: Testing whether promoting parasite-specific $CD4^+$ T cell function via GITR activation improves the outcome of experimental VL

4.1 INTRODUCTION

Combining different anti-parasitic drugs is one way to improve treatment of VL. This approach aims to increase drug efficacy, as well as reduce drug toxicity, parasite resistance and treatment duration and cost [183]. In a recent study carried out by Sunder *et al.* in a cohort of 613 patients, of whom 146 were treated with amphotericin B, 156 with liposomal amphotericin B and miltefosine, 154 with liposomal amphotericin B and paromomycin, and 157 with miltefosine and paromomycin, the combination treatments were more effective, less toxic and better tolerated than treatment with amphotericin B alone [184]. These results demonstrate that combination drug therapy were safer and more effective treatment options for VL [184].

A potential extension of this work could involve combining immune therapy with conventional anti-parasitic drug treatment to optimise control of chronic disease and better protect against re-infection. VL is associated with suboptimal, anti-parasitic $CD4^+$ T cell responses [13, 185]. Experimental studies in VL have emphasized the importance of $CD4^+$ T cell responses in the liver for granuloma formation and control of parasite burden in a TNFα and IFNγ dependent manner [186]. Glucocorticoid-induced TNFα receptor family-related protein (GITR) is expressed at low levels by many immune cells, but is highly expressed on

Foxp3$^+$ Treg cells, and is also up-regulated on conventional CD4$^+$ and CD8$^+$ T cells following activation [187]. A previous study reported that activation of CD4$^+$ T cells during an established experimental *L. donovani* infection with a stimulatory antibody directed against GITR resulted in greatly enhanced anti-parasitic activity and increased CD4$^+$ T cell polarisation to Th1 cells, with minimal effects on Treg cells [114]. Importantly, the agonist anti-GITR mAb acted synergistically with a sub-optimal dose of anti-parasitic drug to improve parasite burdens in both liver and spleen [114].Thus, GITR appears to be a good target for boosting anti-parasitic CD4$^+$ T cell responses.

IL-10 is a major regulatory cytokine produced by leukocytes in response to inflammatory signals during VL. IL-10 production by IFNγ-producing CD4$^+$ T (Tr1) cells have been reported in VL patient spleen and blood samples [46], and studies in IL-10-deficient mice showed that *L. donovani* infection is rapidly controlled, relative to wild type control mice [188]. In addition, blockade of IL-10 signalling during an established infection dramatically enhances anti-parasitic immunity [54]. CTLA-4 (CD152) is a critical T cell regulatory molecule associated with Treg cells. CTLA-4 expression by Treg cells is critical for their ability to suppress immune responses by inhibiting the ability of APCs to activate other T cells, and is now a major target for anti-cancer therapy. Previous studies have shown that antibody blockade of CTLA-4 enhances IFNγ production by T cells in *L. donovani*-infected mice, resulting in better control of parasite growth, hence indicating that IL-10, CTLA-4 and GITR are all good targets for immune modulation to improve treatment for VL. However, these molecules modulate different arms of the host immune response, and it is clear that targeting each of these molecules on their own does not result in complete disease control. Hence, I hypothesise that by combining immune modulation, I can optimise immune

responses to clear parasites more effectively either alone or with conventional anti-parasitic drug treatment.

4.2 **RESULTS**

4.2.1 The effect of combination immune therapy during a chronic *L. donovani* infection

As mentioned earlier (section 2.3.1), experimental VL is characterised by an acute phase in the liver where parasite burdens peak between 2-4 weeks after infection and resolve by weeks 6-8 post infection (p.i.). In contrast, a chronic phase develops in the spleen, with parasite burdens peaking 4 weeks p.i., and persisting for the life of the animal. Given that VL patients generally have chronic infections, I first aimed to improve anti-parasite immune responses using combination immune therapy during the chronic stage of the infection in the spleen. Hence, all treatment was started 28 days p.i.

C57BL/6 mice were infected with *L. donovani* and 28 days later, mice were treated with a single dose of agonist anti-GITR mAb or inhibitory anti-IL-10R or anti-CTLA-4 mAbs. Parasite burdens and cellular analysis were carried out seven days later. Despite a significant reduction following anti-IL-10 mAb administration in the spleen and consistent reductions in parasite burdens following anti-IL-10 or anti-CTLA4 mAb administration in the liver, no antibody treatments were able to significantly improve control of parasite growth in the liver or spleen (apart from anti-IL-10 treatment) (Figure 4.1A and Figure 4.1B). Interestingly, analysis of serum cytokines showed that the group treated with anti-GITR mAb

produced significantly higher amounts of the pro-inflammatory cytokine IFNγ, compared to the other groups (Figure 4.1C).

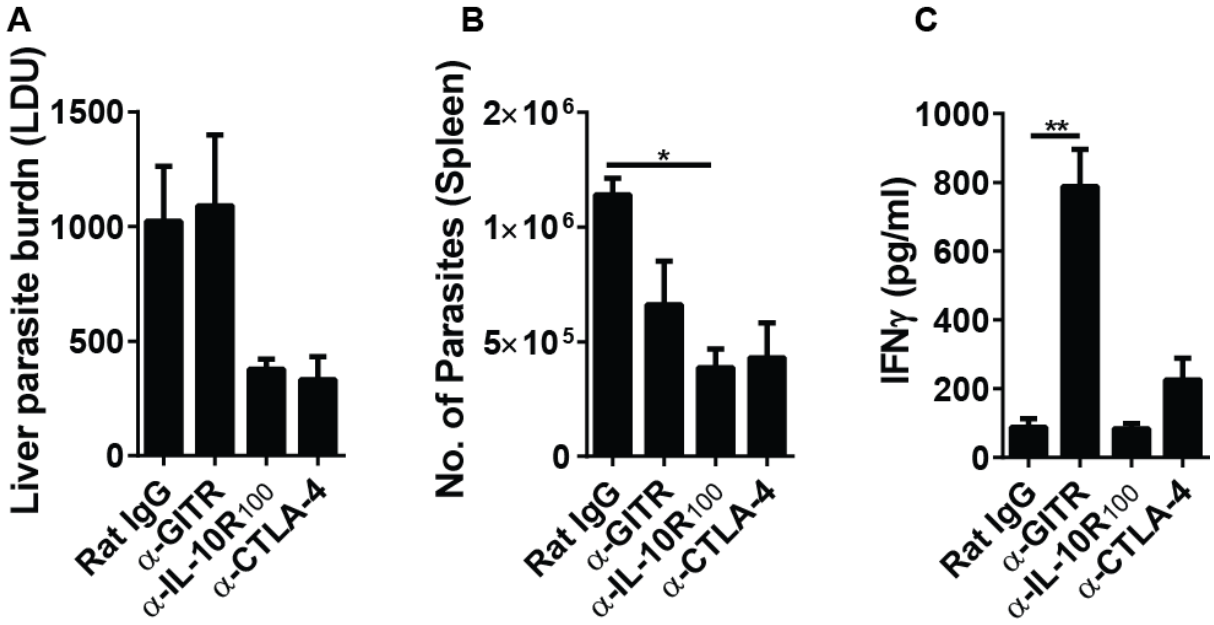

Figure 4.1: Distinct effects of anti-GITR agonist antibody and blocking IL-10R and CTLA-4 interactions on anti-parasitic responses.

Parasite burdens were determined in the (A) livers and (B) spleens of *L. donovani* infected mice treated with anti-GITR mAb or anti-IL-10R mAb or anti-CTLA-4 mAb or control rat IgG on day 28 p.i. (C) IFNγ levels (pg/ml) in serum of treated mice. Data are represented as the mean +/- SEM at day 35 p.i. Statistical differences of $p < 0.05$ (*), $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

Given the above results, I next tested whether treatment with a combination of immune modulators could improve anti-parasitic immunity in this model. C57BL/6 mice were infected with *L. donovani* and 28 days later, mice were treated with various combinations of anti-GITR, anti-IL-10R and anti-CTLA-4 mAbs, Seven days later, groups were assessed for hepatic and splenic parasite burden, and serum cytokine levels were measured.

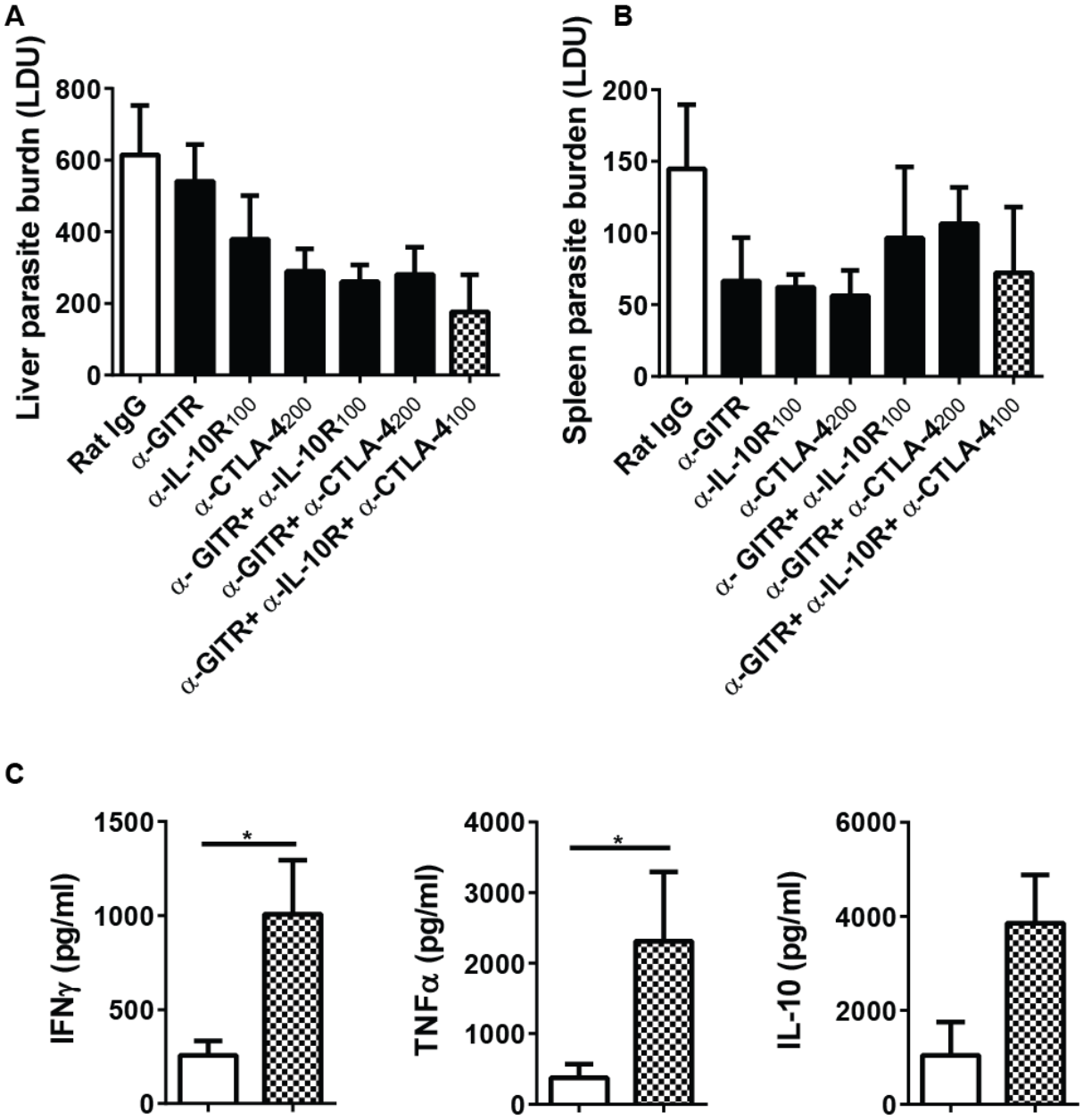

Figure 4.2 : Effects of combination antibody treatment on parasite burdens in liver and spleen.

Parasite burdens were determined in the (**A**) livers and (**B**) spleens of *L. donovani* infected mice treated with mAb alone or a combination of anti-GITR, anti-IL-10R and anti-CTLA-4 mAbs on day 28 p.i. Rat IgG was used as a control. (**C**) IFNγ, TNFα and IL-10 levels (pg/ml) in serum of control mice and mice treated with all 3 test mAbs. Data are represented as the mean +/- SEM at day 35 p.i. Statistical differences of $p < 0.05$ (*) are indicated (n=5 mice per group). Results are representative of a single experiment. [Clear bars = Treated with Rat IgG; Hatched boxes = Treated with combination of anti-GITR, anti-IL-10R and anti-CTLA-4 mAbs]

No single or combined antibody treatment protocol was able to significantly improve control of parasite growth in the liver or spleen, again despite consistent reductions in parasite burden between groups treated with anti-IL-10 and anti-CTLA4 mAbs (Figure 4.2A and 4.2B). Higher levels of the pro-inflammatory cytokine IFNγ and TNFα were measured in the group treated with a combination of all three mAb, compared to the controls, indicative of a strong Th1 response. However, there was a trend for elevated IL-10 levels in the group treated with all three mAbs, compared to the control group (Figure 4.2C).

The above results indicated that no mAb alone had a significant effect on parasite burdens at this time point. To ensure that mice had received a sufficient dose of antibody, the quantity and the number of doses of anti-IL-10 and anti-CTLA4 mAbs was increased from 0.1mg or 0.2mg to 0.5mgs, and mice were treated with three doses of inhibitory antibody instead of one. I still administered a single dose of the anti-GITR mAb, as previous work has shown that multiple dosing was ineffective [114]. Also, instead of combining all three mAbs, the inhibitory antibodies were individually combined with anti-GITR mAb so that I could identify synergistic effects of the anti-GITR with other antibodies.

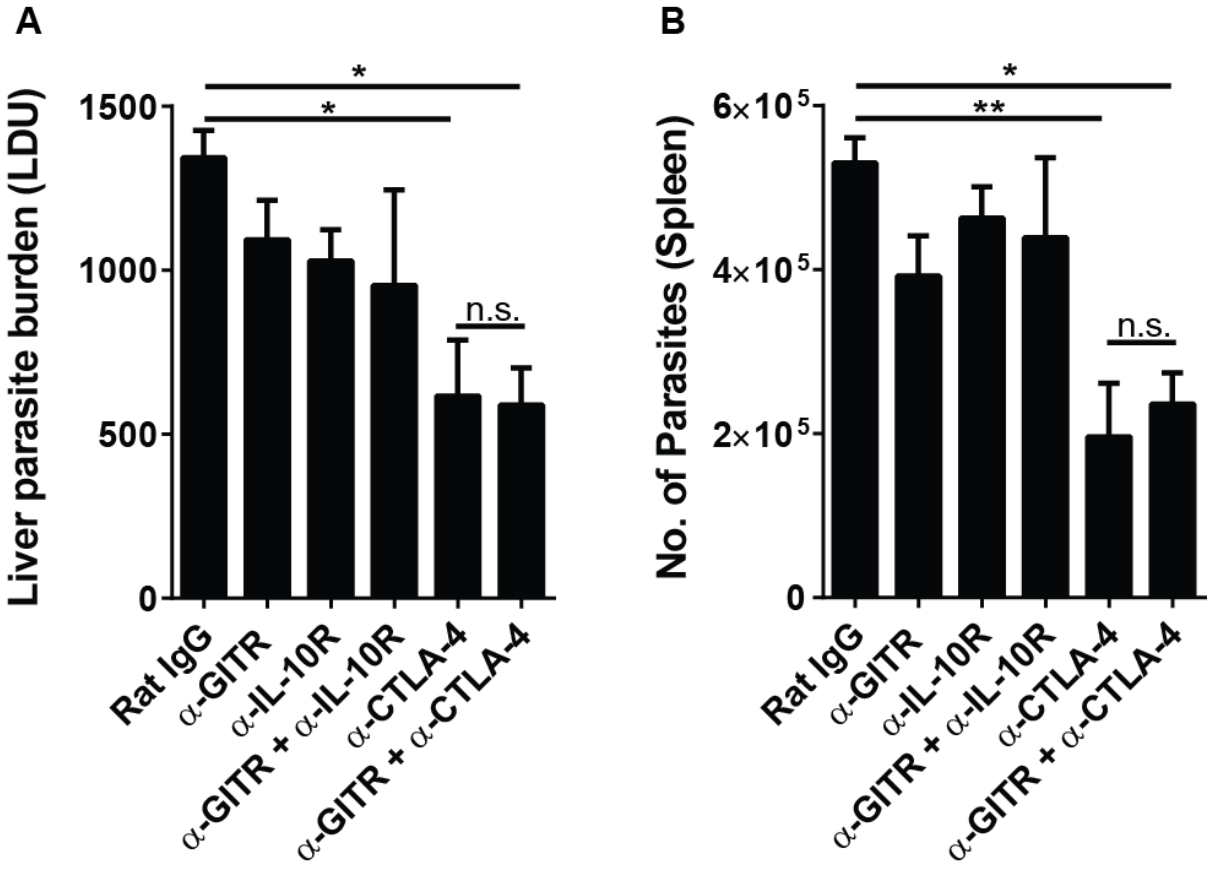

Figure 4.3: Effects of combined antibody treatment on parasite burdens in the liver and spleen.

Parasite burdens were determined in the (**A**) livers and (**B**) spleens of *L. donovani* infected mice treated with the mAb alone or a combination of anti-GITR mAb on day 28 and anti-IL-10R or anti-CTLA-4 mAbs on days 28, 30 and 33 p.i. A dose of rat IgG equivalent to the highest antibody dose was used as a control. Data are represented as the mean +/- SEM at day 35 p.i. Statistical differences of $p < 0.05$ (*) are indicated (n=5-10 mice per group). Results are representative of two different experiments. [n.s. = not significant]

Mice treated with the combination of anti-GITR and anti-IL-10R had no improvement in control of parasite burden in both the liver and the spleen (Figure 4.3), compared to the control groups. Treatment with the combination of anti-GITR and anti-CTLA-4 resulted in decreased parasite burdens in both the liver and the spleen of infected animals. However, combined antibody treatment did not control parasite burdens any better than mice treated with anti-CTLA-4 alone (Figure 4.3). Overall, the data shows that although combination of anti-CTLA4 mAb with the agonist anti-GITR mAb is effective at controlling parasite burden,

it does not improve parasite clearance any more than anti-CTLA4 mAb treatment alone. This data also indicates that increased dosing of anti-CTLA4 mAb improved its anti-parasitic activity. However, the results also suggest that GITR activation has minimal therapeutic potential during the chronic stage of experimental VL.

4.2.2 Effect of combination immune therapy during an acute *L. donovani* infection

From the above experiments, I found that combination mAb therapy during the chronic stage of the *L. donovani* infection did not have any benefit over anti-CTLA4 or anti-IL-10 mAbs alone on control of parasite burdens in the organs of infected mice. Therefore, I changed my attention to the acute phase of the infection, with the aim to improve immune responses in the liver before infection became established in the spleen. As with previous studies, I found that combining anti-CTLA-4 with anti-GITR mAbs had no additive effect on parasite control. Therefore, I focused on using only the anti-IL-10R inhibitory mAb, since past work suggests that IL-10 is potent suppressor of cell mediated immunity and blocking of IL-10 improved cellular immune responses both in experimental VL and human VL [46, 54, 188].

C57BL/6 mice were infected with *L. donovani* for 14 days, and then treated with the inhibitory anti-IL-10R mAb and agonistic anti-GITR mAb individually or in combination. Parasite burdens and cellular analysis were carried out 14 days later (day 28 p.i.). The anti-IL-10 mAb was given at a dose of 0.5mg, 3 times, while anti-GITR mAb was administered at a single dose at day 14 p.i.

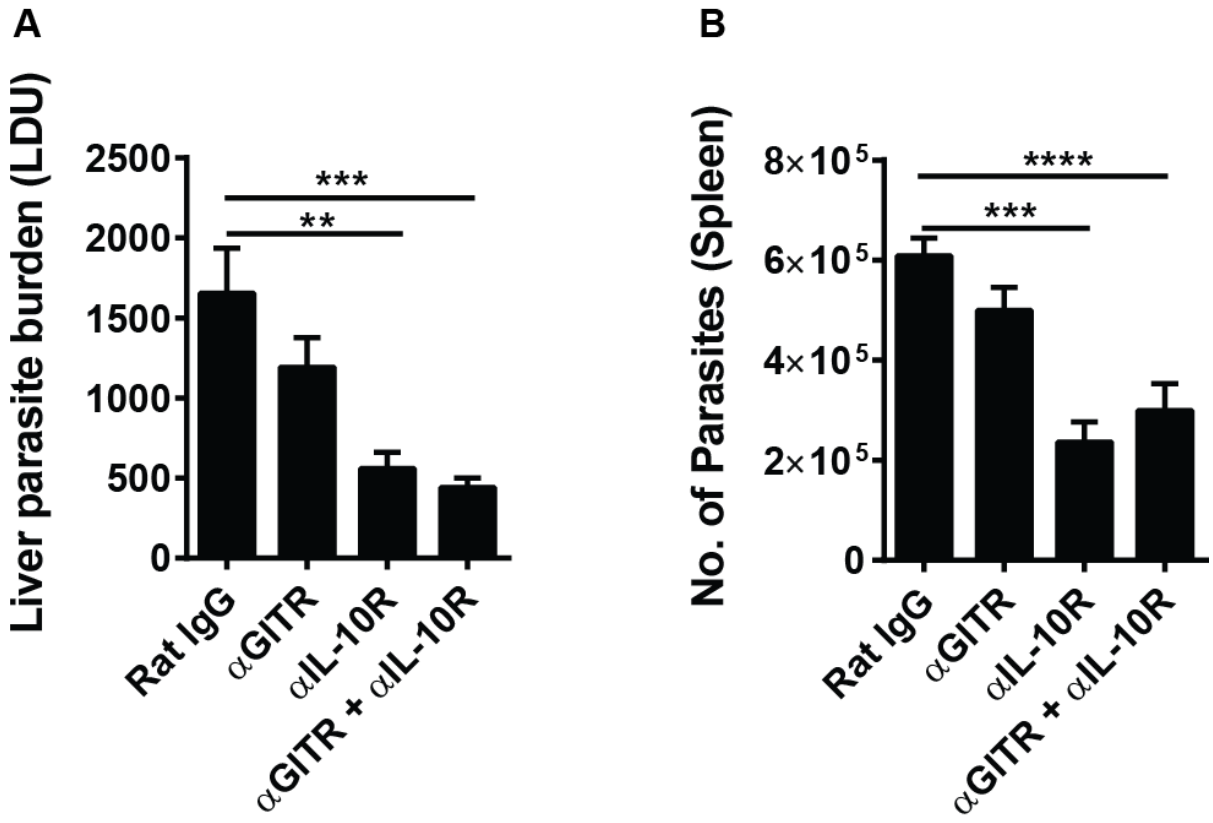

Figure 4.4: Effects of combination antibody treatment on parasite burdens during acute infection.

Parasite burdens were determined in the (**A**) livers and (**B**) spleens of *L. donovani* infected mice treated with the mAb alone or a combination of anti-GITR mAb and anti-IL-10R mAb on day 14, 19 and 24 p.i. Rat IgG was used as a control. Data are represented as the mean +/- SEM at day 28 p.i. Statistical differences of $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) are indicated (n=15-17 mice per group). Results are representative of three different experiments.

Mice treated with the combination of anti-GITR and anti-IL-10R mAbs showed statistically significant improvement in control of parasite burden in both the liver and the spleen (Figure 4.4), compared to the control group. However blocking IL-10 alone was just as effective, indicating no additive effect of GITR activation.

In a repeat experiment set up to confirm the results, mice were accidently infected with a four times lower dose of parasite inoculum (5×10^6 amastigotes i.v.), and results from this experiment showed that combination anti-GITR and anti-IL-10R mAb had a detrimental

effect on anti-parasitic immunity in the liver (Figure 4.5). This result was confirmed by investigating the effects of antibody treatments on mice infected with a lower parasite inoculum ($5x10^6$ parasite/mouse). Mice infected with lower parasite numbers had consistently reduced parasite burdens following GITR activation, although this did not reach statistical significance. IL-10 blockade resulted in significantly decreased parasite burdens, compared to the control group, but again, combining this treatment with GITR activation resulted in increased parasite burdens in the liver (Figure 4.5). Thus, GITR activation was antagonistic to the anti-parasitic effects of IL-10 blockade.

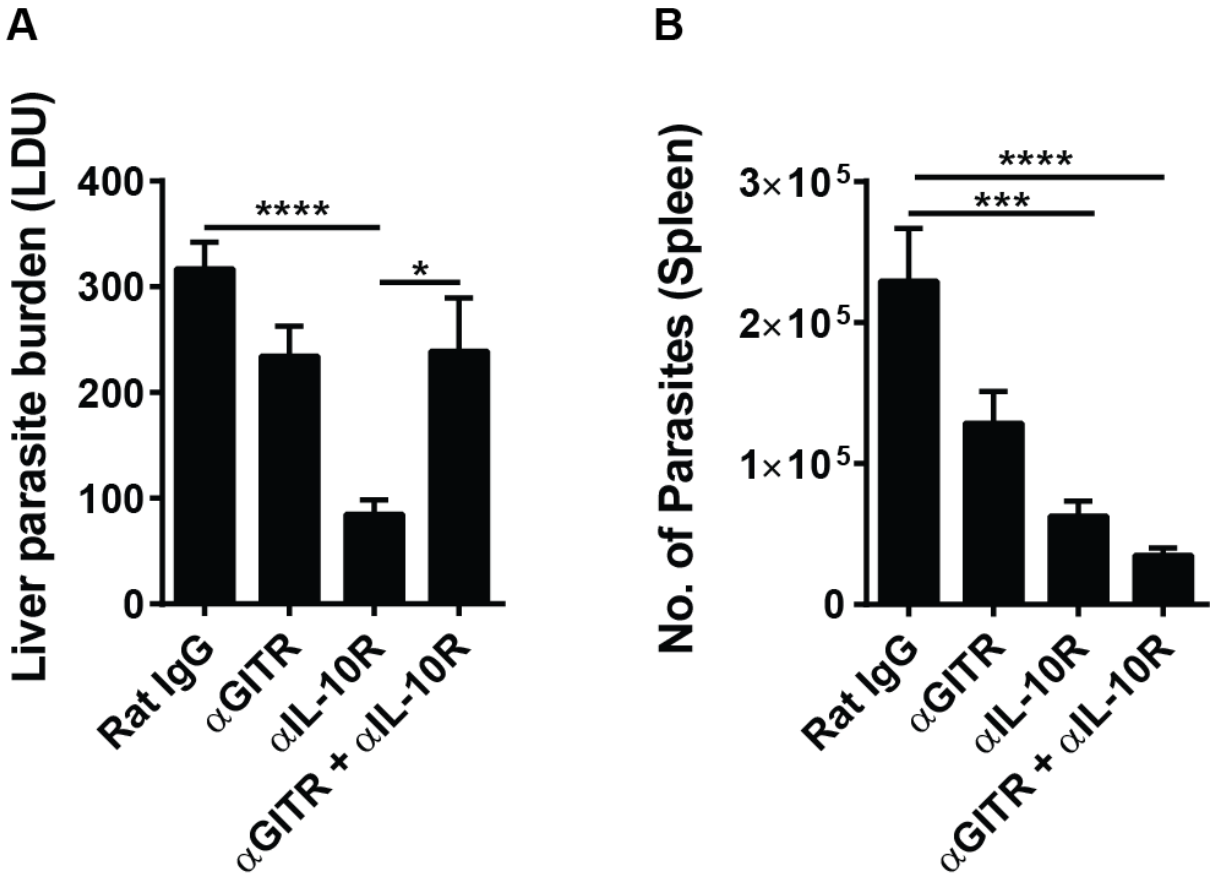

Figure 4.5: The dose of infection determines combination mAb treatment outcome.

Parasite burdens were determined in the livers (**A**) and spleens (**B**) of mice infected with the low dose of parasite inoculum. Infected mice were treated with mAb alone or a combination of anti-GITR mAb and anti-IL-10R mAb, as indicated. Rat IgG was used as a control. Data are represented as the mean +/- SEM at day 28 p.i. Statistical differences of $p < 0.05$ (*), $p < 0.001$ (***) and $p < 0.0001$ (****) are indicated (n=15 mice per group). Results are representative of three different experiments.

As an extension of this work, our collaborator in India also examined the effect of combined IL-10 blockade and GITR activation in VL patient samples (Appendix 1).This data suggested that the low dose experimental VL model in the acute stage of infection might better represent the immune environment in human VL patients because combined antibody treatment resulted in similar outcomes, whereby GITR activation was antagonistic to the anti-parasitic effects of IL-10 blockade. Given this result, I focused my efforts studying the impact of antibody treatments on liver responses in the low dose infection model.

4.2.3 Effect of combined anti-GITR mAb and anti-IL-10 mAb therapy on immune parameters during a low-dose *L. donovani* infection

I next examined the frequency and number of Th1 ($Tbet^+$ IFNγ-producing $CD4^+$ T cells) cells, Tr1 (IL-10 and IFNγ producing $CD4^+$ T cells) cells, and terminally differentiated Th1 ($Tbet^+$ $KLRG\text{-}1^+$ $CD4^+$ T cells) cells in different treatment groups. Initially, this was carried out as a comparative study between the high dose (2×10^7 per mouse) and low dose (5×10^6 per mouse) infection, and both the liver and the spleen of infected mice were analysed (Figure 4.6).

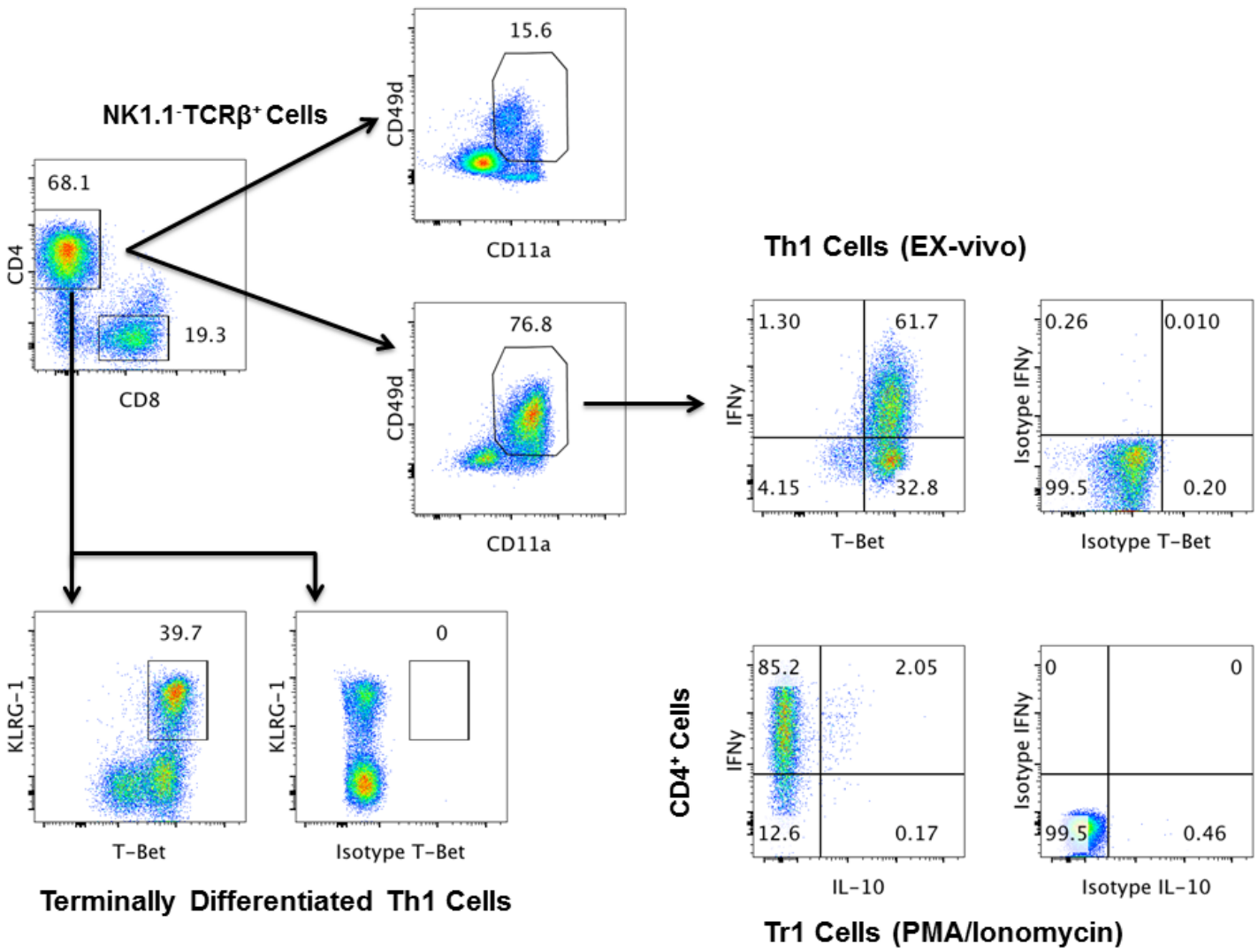

Figure 4.6: Representative sequential gating strategy for the isolation of Th1 cells, Tr1 cells and terminally differentiated $CD4^+$ T cells.

From the $TCR\beta^+$ NK 1.1^- cell fractions, $CD4^+$ and $CD8^+$ cells were gated. Activated $CD4^+$ T cells were selected based on CD49d and CD11a surface markers. Intracellular staining was carried out on activated $CD4^+$ T cells, to identify Th1 ($Tbet^+$ IFNγ-producing $CD4^+$ T cells) cells. Tr1 (IL-10- and IFNγ-producing $CD4^+$ T cells) cells, and terminally differentiated Th1 ($Tbet^+$ $KLRG\text{-}1^+$ $CD4^+$ T cells) cells from the liver $CD4^+$ T cell population.

$CD4^+$ T cell analysis carried out on liver cells showed only minor difference in the frequency and number of Th1 (Figure 4.7 A and B) and Tr1 (Figure 4.8 A and B) cells between mice treated with either agonistic anti-GITR or inhibitory anti-IL-10R mAbs or a combination of both, irrespective of the dose used to establish infection (i.e., low dose (Figure 4.7A and Figure 4.8A) or high dose (Figure 4.7B and Figure 4.8B)).

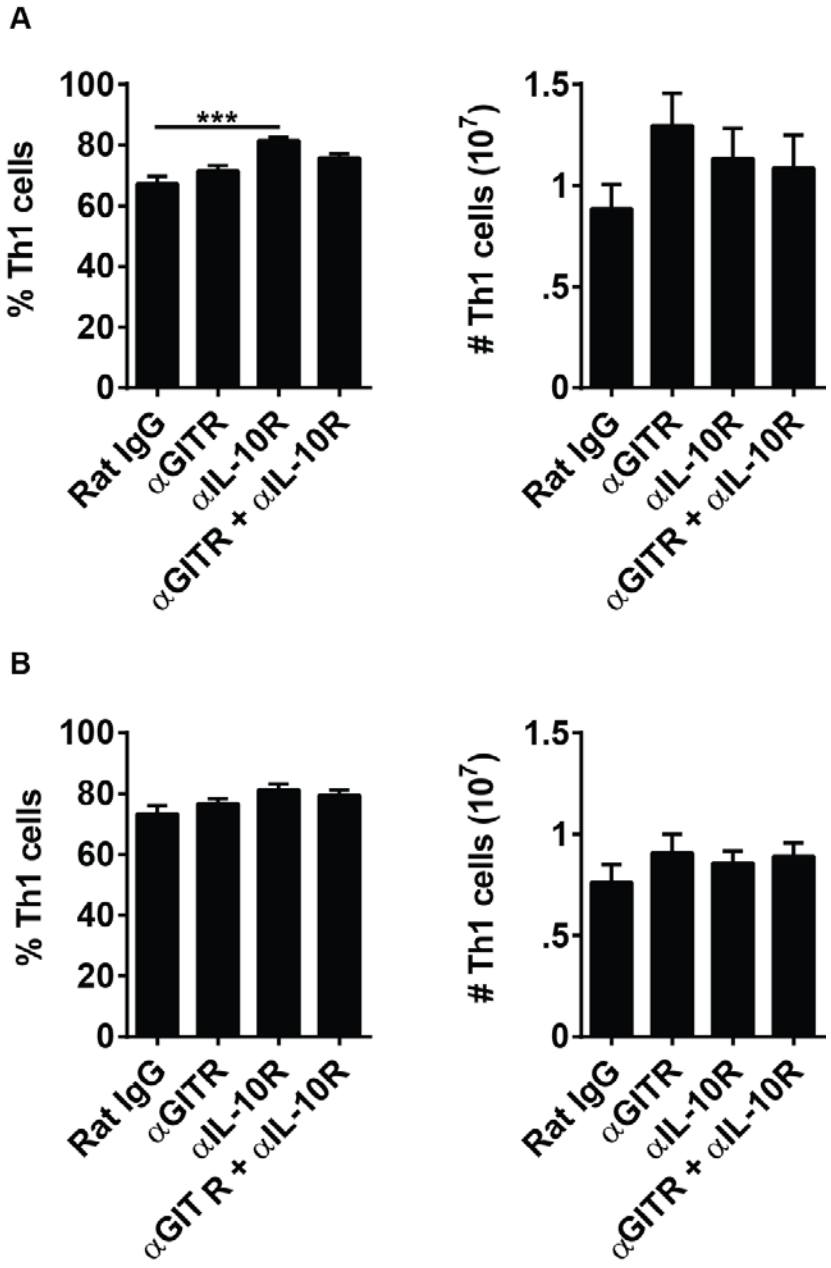

Figure 4.7: Immune modulation has little effect on Th1 responses in the liver.

Hepatic Th1 cellular responses were measured in mice infected with *L. donovani* (a low (A) or high (B) dose of inoculum) treated with the mAb alone or a combination of anti-GITR mAb and anti-IL-10R mAb on days 14, 19 and 24 p.i. Rat IgG was used as a control. Both the frequency and total number of Th1 cells are shown graphically. Data are represented as the mean +/- SEM at day 28 p.i. statistical differences of $p < 0.0001$ (****) are indicated (n=15 mice per group). Results are representative of three different experiments.

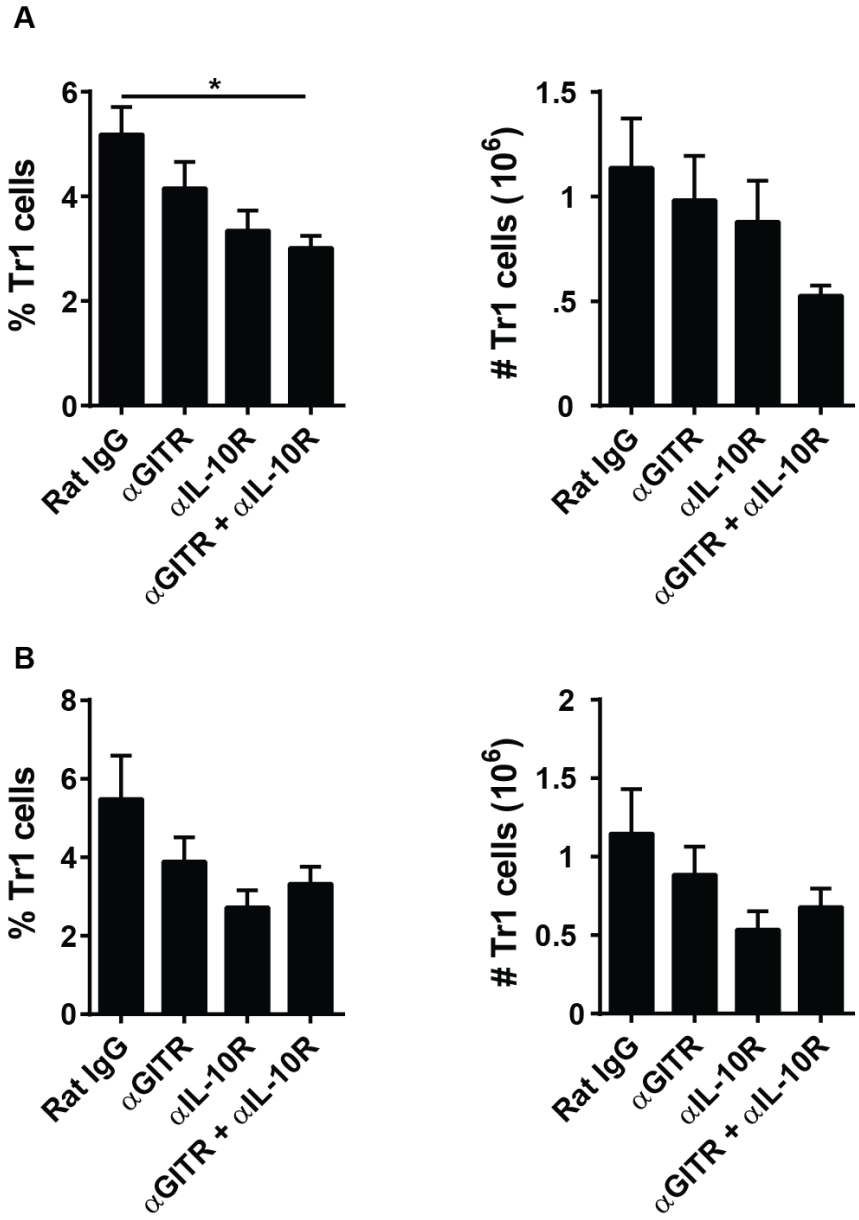

Figure 4.8: Immune modulation has little effect on Tr1 responses in the liver.

Hepatic Tr1 cellular responses were measured in mice infected with *L. donovani* (a low (A) or high (B) dose of inoculum) treated with the mAb alone or a combination of anti-GITR mAb and anti-IL-10R mAb on days 14, 19 and 24 p.i. Rat IgG was used as a control. Both the frequency and total number of Tr1 cells are shown graphically. Data are represented as the mean +/- SEM at day 28 p.i. (n=15 mice per group). Results are representative of three different experiments.

Antigen experienced cells that had become terminally differentiated were identified by expression of KLRG-1 (Killer cell lectin-like receptor G1) [189]. Cells expressing the KLRG-1 marker have diminished proliferative capacity and are functionally exhausted [190]. Terminally differentiated Th1 cells were identified by expression of KLRG-1 and Tbet on $CD4^+$ T cells. In infected mice treated with the combination of anti-GITR and anti-IL-10R mAbs, a significant increase in the frequency of KLRG-1 expressing Th1 cells was observed in the liver, compared to the control mice given Rat IgG (Figure 4.9A and B).

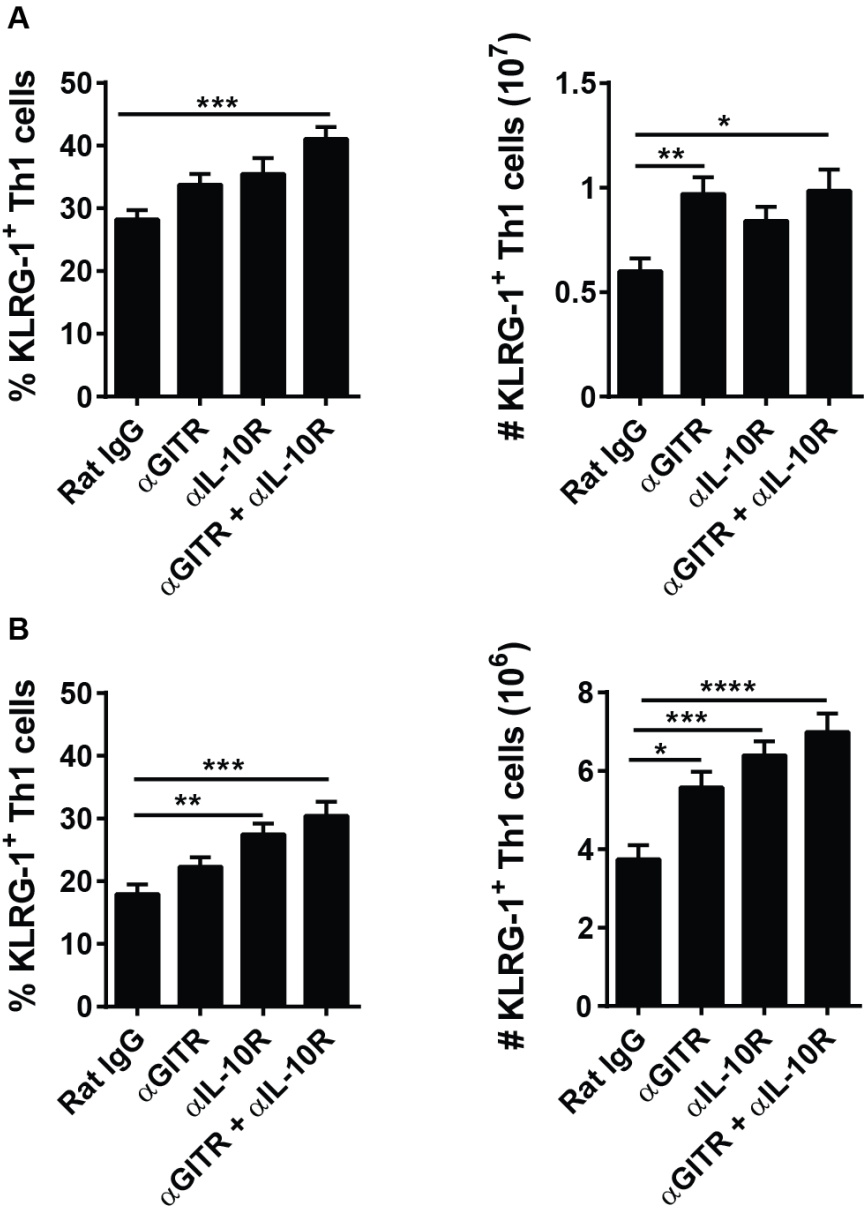

Figure 4.9: Increased frequency and number of terminally differentiated hepatic Th1 cells in groups treated with combined anti-GITR and anti-IL-10R mAbs.

Hepatic KLRG-1$^+$ Th1 cellular responses were measured in mice infected with *L. donovani* (a low (A) or high (B) dose of inoculum) treated with the mAb alone or a combination of anti-GITR mAb and anti-IL-10R mAb on days 14, 19 and 24 p.i. Rat IgG was used as a control. Both the frequency and total number of KLRG-1$^+$ Th1 cells are shown graphically. Data are represented as the mean +/- SEM at day 28 p.i. statistical differences of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) are indicated (n=15 mice per group). Results are representative of three different experiments.

Further analysis revealed that mice inoculated with the lower dose of parasites had a significant increase in the frequency and number of terminally differentiated Th1 cells compared to mice infected with a higher dose. This difference was observed in the liver across all treated groups (Figure 4.10 A and B).

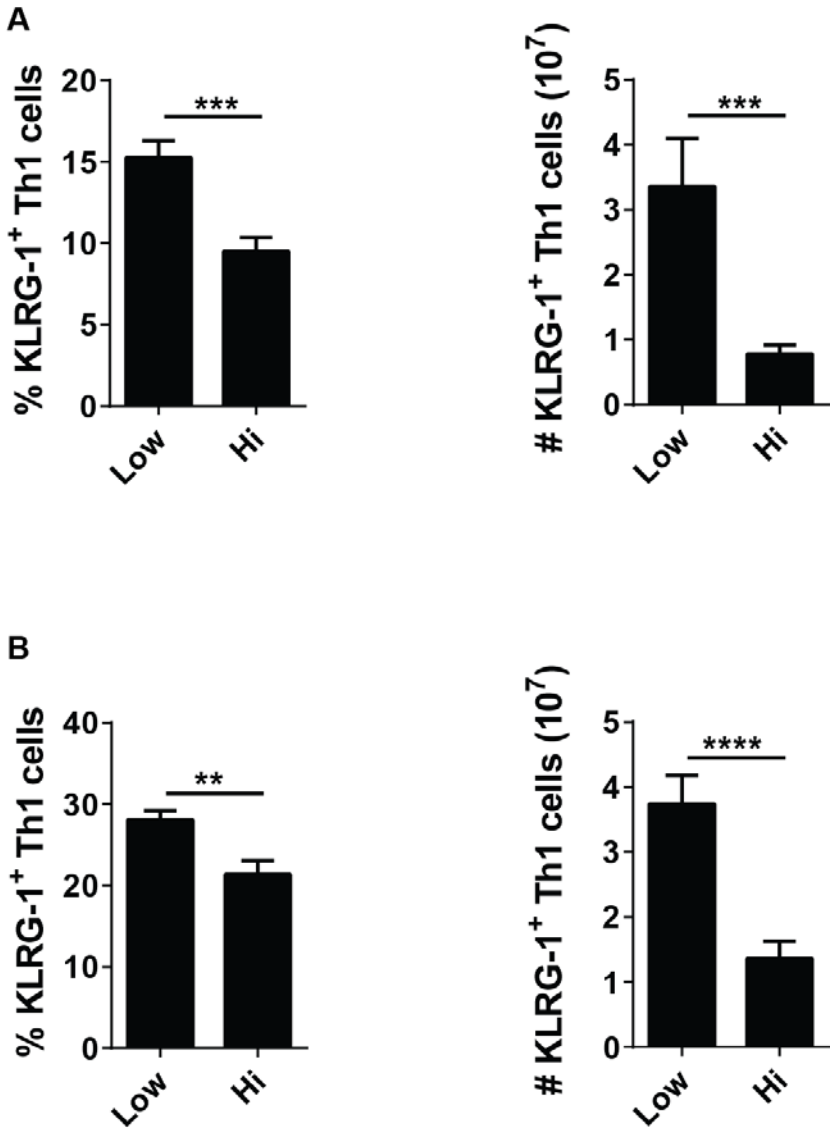

Figure 4.10: Increased number and frequency of terminally differentiated hepatic Th1 cells in mice infected with low numbers of parasites.

Hepatic KLRG-1$^+$ Th1 cellular responses were measured in mice infected with *L. donovani* (a low or high dose inoculum). (A) Frequency and number of KLRG-1$^+$ Th1 cell in mice treated with control Rat IgG. (B) Frequency and number of KLRG-1$^+$ Th1 cell in mice treated with both anti-GITR and anti-IL-10R mAbs. Data are represented as the mean +/- SEM at day 28 p.i., and statistical differences of $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) are indicated (n=15 mice per group). Results are representative of three different experiments.

These results show that while in a low dose infection I observed an increased Th1 response, these cells have a more functionally exhausted phenotype compared to the corresponding groups in mice infected with higher parasite numbers. This phenotype

increased when infected mice were treated with the combination of anti-GITR and anti-IL-10R mAbs (Figure 4.10 A and B).

4.2.4 Effect combining immune therapy with drug treatment on an *L. donovani* infection

As mentioned earlier, combination drug therapy appears to be a promising therapeutic method for the treatment of active VL. Hence I wanted to assess the impact of combining immune modulation with drug therapy as a way to improve treatment outcome. A sub-optimal dose of sodium stibogluconate (SSG Sub) was used to try and identify any positive anti-parasitic effects from mAb treatments.

C57BL/6 mice infected with a low dose of *L. donovani* parasite and 14 days later were treated with a sub-optimal dose of drug (SSG Sb) and then every two days after that for 14 days. Treatment with combination inhibitory anti-IL-10R antibody and agonistic anti-GITR antibody was carried out as described above (e.g. Figure 4.9). The combination of drug therapy and immune modulation significantly reduced parasite burden in both organs, when compared to control groups (Figure 4.11 A and B).

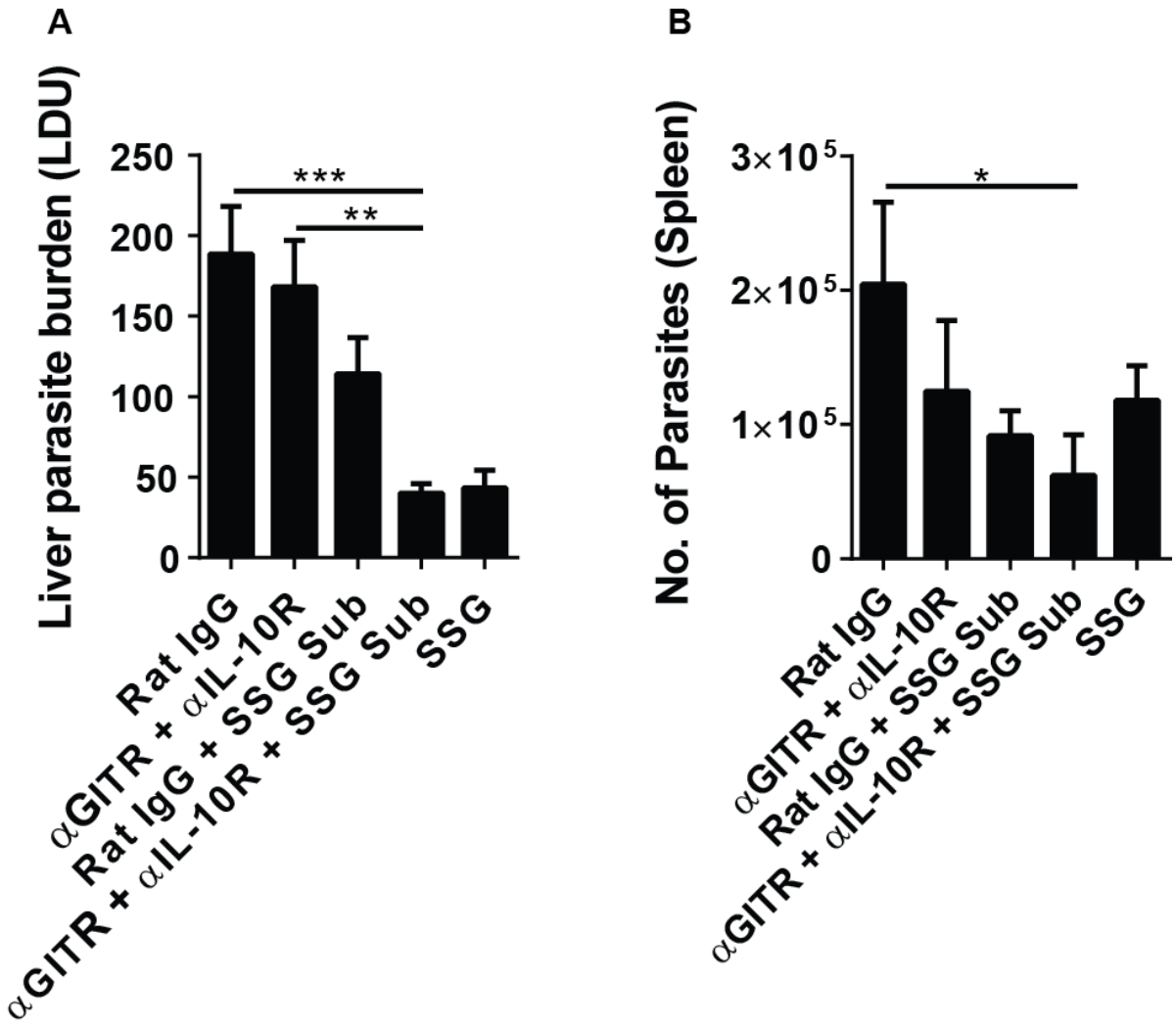

Figure 4.11: Immune modulation combined with sub-optimal drug therapy improved control of parasite burden.

L. donovani infected mice were treated either with control rat IgG or with a combination of both anti-GITR mAb and anti-IL-10R mAb in the presence or absence of SSG Sub. (A) Hepatic parasite burden and (B) Spleen parasite burdens, respectively, are shown. Data are represented as the mean +/- SEM at day 28 p.i., and statistical differences of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) are indicated. (n=10 mice per group). Results are representative of two different experiments.

Analysis of Th1 cells from the livers of mice treated with immune modulators and the sub-optimal dose of drug showed no significant differences, compared to the control groups (Figure 4.12 A). However the number of KLRG-1+ Th1 cells significantly decreased in groups treated with the sub-optimal dose of drug (Figure 4.12 B). This indicated that while

drug treatment along with immune modulation does not alter the development of Th1 cells, it does reduce the rate of conversion of these cells to a functionally exhausted phenotype.

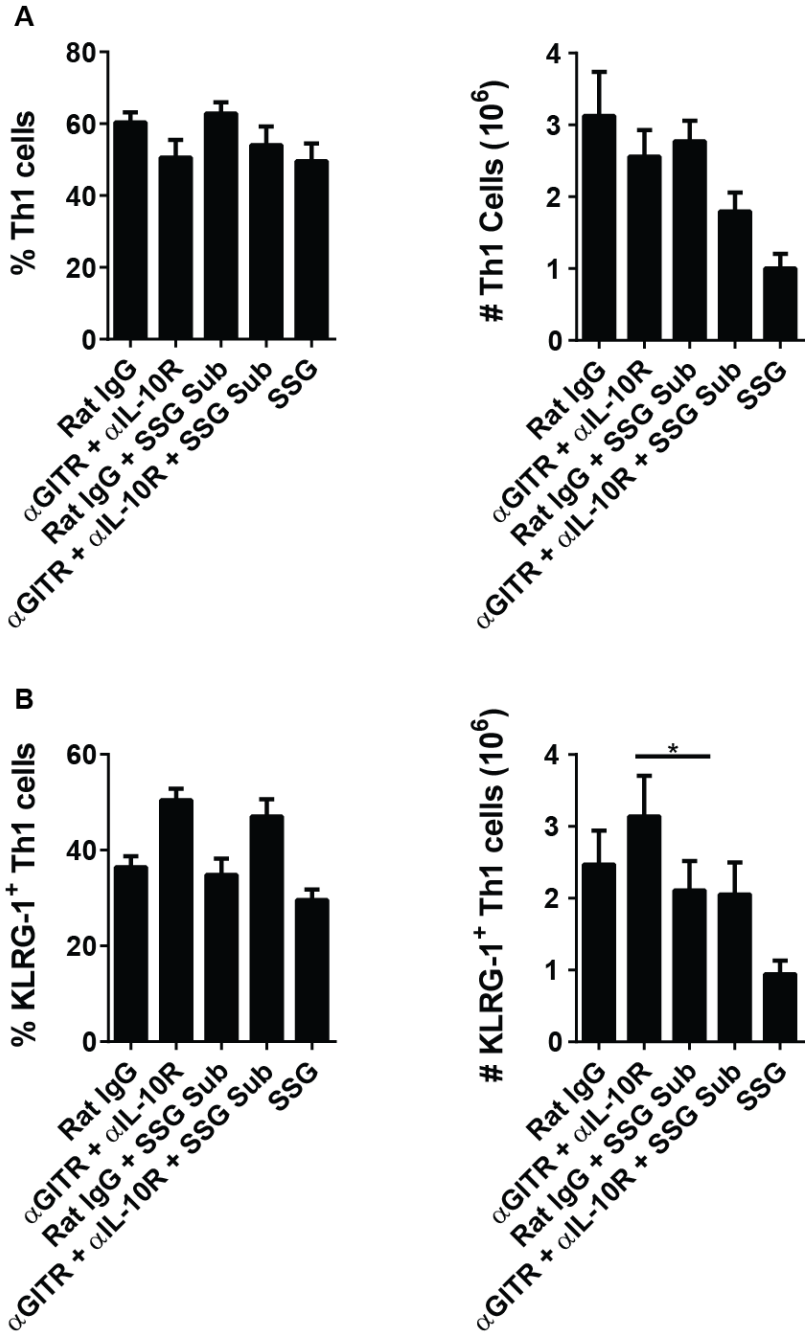

Figure 4.12: Combined mAb administration with drug treatment reduces the number of terminally differentiated Th1 cells.

Hepatic Th1 (**A**) and KLRG-1$^+$ Th1 (**B**) cellular responses were measured in mice infected with a low dose of *L. donovani* . Mice were treated a combination of anti-GITR mAb and anti-IL-10R mAb on days 14, 19 and 24 p.i., with or without drug. Rat IgG was used as a control. Both the frequency and total number of Th1 and KLRG-1$^+$ Th1 cells are shown graphically. Data are represented as the mean +/- SEM at day 28 p.i., and statistical differences of $p < 0.05$ (*) are indicated. (n=10 mice per group). Results are representative of two different experiments.

Antigen-specific cellular immune responses were also measured after treatment, and an increase in the frequency of antigen-specific $CD4^+$ $IFN\gamma^+$ $TNF\alpha^+$ cells was found in mice treated with combination antibody and drug, compared to mice given control antibody (Figure 4.13A). Cytokine analysis cell culture supernatants showed a significant increase in the levels of IFNγ and TNFα, but not IL-10, in spleen cells from mice treated with combination antibody and drug, compared to the control (Figure 4.13B).

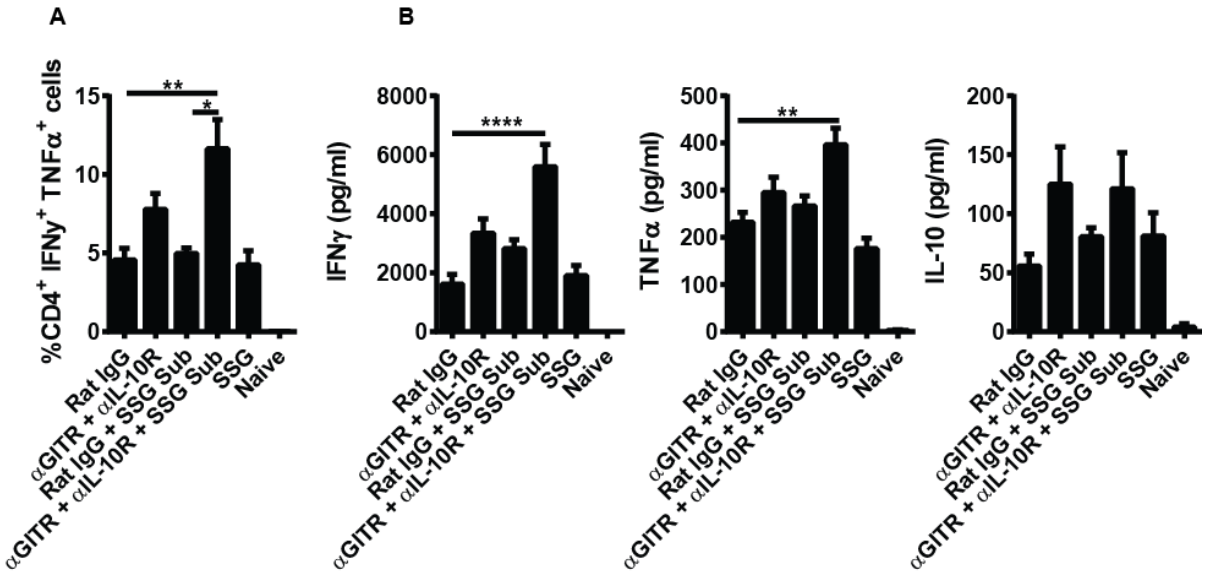

Figure 4.13: Antigen-specific cellular immune responses after combined mAb administration and drug treatment.

Spleen cells isolated from *L. donovani* infected mice treated with control rat IgG or with a combination of anti-GITR and anti-IL-10R mAbs in the presence or absence of drug were cultured with parasite antigen for 72 hrs. (**A**) Frequency of $CD4^+$ $IFN\gamma^+$ $TNF\alpha^+$ T cells and (**B**) IFNγ, TNFα and IL-10 levels in cell culture supernatants were measured. Data are represented as the mean +/- SEM at day 28 p.i., and statistical differences of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.0001$ (****) are indicated. (n=10 mice per group). Results are representative of two different experiments.

These results indicate that the addition of drug to combination immune therapy can reverse the adverse effect of GITR activation on IL-10R blockade, and also significant improve anti-parasite $CD4^+$ T cells responses.

To establish whether combined antibody was better than single antibody treatment when used with drug, mice infected with a low dose of *L. donovani*, and treated with anti-GITR and anti-IL-10R or a combination of both, with or without a sub-optimal dose of drug. Sub-optimal drug treatment reduced hepatic parasites burdens in all antibody treated groups, but only reached statistical significance when combination therapy was used in conjunction with drug treatment, as seen previously (Figure 4.14A). Assessment of antigen-specific cellular immune responses showed that drug treatment with combination therapy as well as with anti-IL-10R alone, resulted in significant increase in IFNγ and TNFα in cell culture supernatants of antigen-stimulated spleen cells (Figure 4.14B).

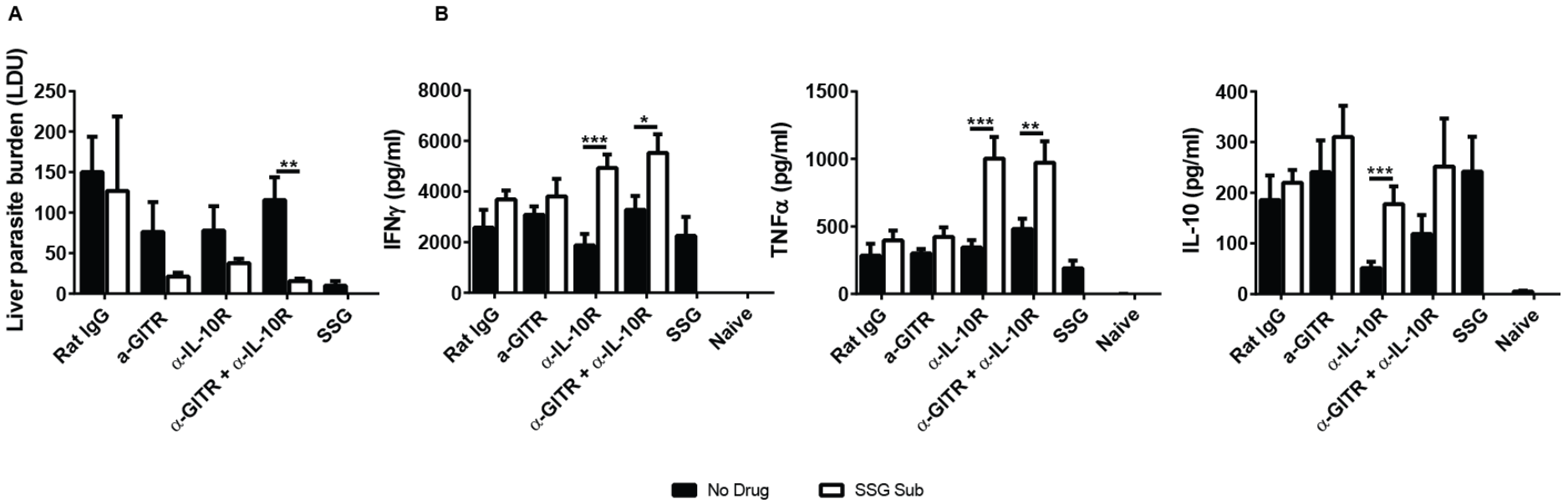

Figure 4.14: Anti-parasitic immune responses after combined mAb therapy and sub-optimal drug treatment.

(**A**) Liver parasite burden at day 28 p.i. (14 days after the start of treatment) is shown. (**B**) Splenic cells isolated from *L. donovani* infected mice were treated either with control rat IgG or with a combination of both anti-GITR and anti-IL-10R mAbs in the presence or absence of drug were cultured with parasite antigen for 72 hrs. IFNγ, TNFα and IL-10 levels were measured in cell culture supernatant. Data are represented as the mean +/- SEM at day 28 p.i., and statistical differences of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) are indicated. (n=6 mice per group). Results are representative of a single experiment.

4.3 **DISCUSSION**

In this Chapter, the potential of combining immune modulators to improve experimental VL disease outcome was examined. To achieve this, agnostic anti-GITR and inhibitory anti-IL-10R and anti-CTLA-4 mAbs were used. Previous experiments have shown that GITR is a potential therapeutic target for the treatment of VL [114], and IL-10 and CTLA-4 blockade both improve anti-parasitic immune responses [105, 191]. Hence, *L. donovani* infected mice were treated with different combinations of agnostic anti-GITR and inhibitory anti-IL-10R and anti-CTLA-4 mAbs.

The spleen in VL infected individuals as well as in experimental VL is the site of a chronic and persistent infection. Initially, I aimed to use combination immune therapy to treat this targeted organ to reduce parasite load by improving anti-parasitic immune responses. Hence treatment with the immune modulators was conducted 28 days p.i. However, I found little indication that any mAb treatment either alone or in combination with other mAbs had any statistically significant effect on parasite burdens. Hence, these results indicated that the chronic stage of infection wasn't the ideal setting to assess the potential of combining immune modulators for the treatment of VL. Indeed, I found that the immune environment in the livers of mice at day 14 p.i., infected with a lower parasite inoculum (Figure 4.5), seemed to better reflect the anti-parasitic immune environment in VL patients (Appendix 1). In both settings, GITR activation suppressed improved anti-parasitic immunity following IL-10 blockade. In the low dose mouse model, this was associated with a dramatic increase in the frequency of functionally exhausted Th1 cells, but this negative effect of GITR activation could be reversed by including drug treatment. Studies by our Indian collaborators showed

that improved anti-parasitic cellular responses to anti-IL-10 mAb treatment were no longer suppressed by GITR activation after drug therapy, but were not improved above control-treated samples (Appendix 1). Together, these data indicate that targeting GITR activation offers no significant benefit over IL-10 blockade, and may actually reverse some of the positive effects of anti-IL-10 treatment. My finding identified several factors that impacts the effectiveness of immune modulation, including the parasite burden, tissue target and anti-parasitic drug used. It also highlights the adverse effect of combining immune modulation strategies.

In recent years, patients with VL generally present themselves at the clinic earlier during disease progression, due to the increased awareness of the disease and improved treatment programs aimed at VL eradication [192]. Thus it is possible that immune responses observed in early clinical VL closely resembles liver anti-parasitic responses in the low parasite burden setting of the experimental VL. Another reason the low dose liver model is better as it may closely reflect the disease in cured VL patients that still retain persistent parasite and asymptomatic *L. donovani* infected individuals. These individual have low parasite burdens and are reservoirs of *Leishmania* parasites thus play a role in anthroponotic transmission [193].

A direct correlation between parasite load and IL-10 producing Tr1 cells has been reported in human VL [46, 194]. Tr1 cells have a suppressive effect on anti-parasitic immune responses namely Th1 activation in both experimental and human VL [32, 105]. In my experiments mice infected with low parasite inoculum, low Tr1 responses were observed. This however did not result in enhanced Th1 responses, but I found evidence of increased

Th1 cellular exhaustion in response to combined antibody treatment. Thus it appears that combined activation of GITR and IL-10 blockade stimulated Th1 cell expansion and subsequent exhaustion, with no improvement in anti-parasitic immune responses.

The recent studies in the Indian subcontinent, using a single-dose of liposomal amphotericin B treatment in VL patients, showed a 95.7 % efficiency rate, with low side effects in treated individuals [164, 195], thus questioning the need for immune-therapy. Although the incidence of drug-resistance developing against the single dose treatment is low [164] it is still a possibility. Therefore I think that combining immune therapy with drug therapy is required to further sustain immune responses once targeted elimination by drug therapy have been meet.

As described earlier several immune check point inhibitors have been identified, that could be targeted and combined with drug for beneficial out comes. Recent studies have shown that combining anti-CTLA-4 and anti-PD-1 mAbs has superior anti-tumour immune responses and drastically improves clinical outcome [196]. Alternatively PD-1 blockade which alone improves immune responses against malaria, combined with agonistic anti-OX40 mAb in mice infected with *Plasmodium yoelii* resulted in excessive T cell IFNγ production that negatively influenced anti-parasitic antibody production [197]. These studies together with my study indicates that development of immunotherapy strategies whether as a single or combined treatment, have the potential to be valuable therapies in various disease elimination programs', however identifying the right targets without causing harm to the host is the key.

In conclusion, my study shows that IL-10 blockade alone is superior to GITR activation either alone or in combination. In in a low parasite burden setting GITR activation has an antagonist effect when combined with IL-10 blockade and this effect can be reversed with an anti-leishmanial drug. The low dose experimental VL model best represents the immune environment in VL patients in the Indian subcontinent, and should considered for further therapeutic strategies against VL.

Chapter 5: To test whether IL-2 signalling pathways are deficient in T cells during VL and to test the ability of IL-2/anti-IL-2 mAb complexes to treat and improve experimental VL outcome.

5.1 INTRODUCTION

Interleukin 2 (IL-2) is produced by $CD4^+$ and $CD8^+$ T cells following activation by antigen and is required for proliferation, differentiation and homeostasis of $CD4^+$ T cells, $CD8^+$ T cells and NK cells [198, 199]. IL-2 acts on cells by binding to either the high affinity trimeric IL-2 receptor (IL-2R) made up of IL-2Rα (CD25), IL-2Rβ (CD122) and IL-2Rγ (CD132) or the dimeric IL-2R (comprising β and γ chains) [198]. The trimeric IL-2R receptor is highly expressed on activated $CD4^+$ T cells and $CD4^+$ T regulatory (Treg) cells expressing the forkhead box P3 gene (FoxP3), while memory $CD8^+$ T cells and NK cells express high levels of the dimeric IL-2R [198].

The ability of IL-2 to promote T and NK cell responses makes it an attractive molecule for immunotherapy. Work by Murray *et al.* showed that *L. donovani* infected mice receiving IL-2 blocking mAb failed to control parasite growth in the liver, associated with impaired granuloma development, compared to control mice [200]. Treating *L. donovani* infected mice with exogenous IL-2, however, resulted in > 50% reduction in liver parasite burdens and an increase in the formation of granulomas [200]. Various other studies have shown that therapy with exogenous IL-2 resulted in expansion of $CD4^+$ T cells in chronic viral infection [201]

and improved survival rates in the patients with renal carcinomas and malignant melanomas [202]. However, the therapeutic application of IL-2 has limitations because IL-2 has a short half-life and is rapidly cleared from circulation via the kidneys. Administering high doses of IL-2 to overcome this drawback has serious and adverse side effects such as vascular leak syndrome (VLS), which affects the liver and the lungs leading to liver damage and pulmonary oedema [203].

Recent studies have shown that combining recombinant IL-2 with certain IL-2-reactive monoclonal antibodies (mAbs) can preserve IL-2 signalling capacity and enhance the half-life of IL-2 *in-vivo* (Figure 5.1) [204]. Additionally different IL-2 mAbs expose different IL-2R binding sites when bound to recombinant IL-2 [205]. For example, injecting the S4B6 anti-IL-2 mAb in complex with IL-2 (IL-2Sc) into mice resulted in enhanced stimulation and expansion of $CD8^+$ T cell and NK cell populations, but had little or no effect on $CD25^+$ T cells [205]. In contrast, injecting IL-2 conjugated to the JES6.1A12 anti-IL-2 mAb (IL-2Jc) into mice led to selective stimulation and expansion of $CD25^+$ T cells, but not $CD25^-$ T cells, and the $CD25^+$ T cells were mainly $Foxp3^+$ Treg cells [205].

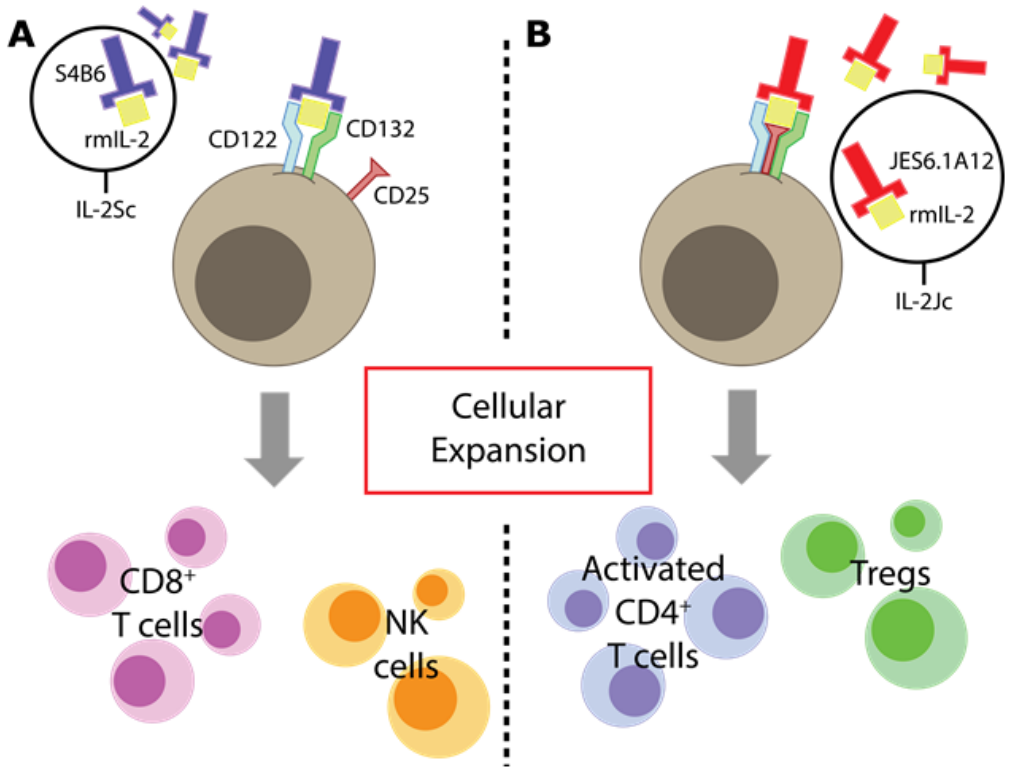

Figure 5.1: IL-2/anti-IL-2 mAb complexes selectively stimulate lymphocyte subsets.

Depending on the type of IL-2 neutralizing monoclonal antibody used, recombinant IL-2 cytokine can be selectively directed to $CD25^-$ cells, which express the low affinity IL-2Rs (**A**) or to $CD25^+$ cells that express high affinity IL-2Rs (**B**). The S4B6 mAb complexed with IL-2 (IL-2Sc) selectively targets cells expressing the low affinity receptors, such as $CD8^+$ T cells and NK cells (**A**), while the JES6.1A12 mAb complexed with IL-2 (IL-2Jc) targets $CD25^+$ cells, such as activated $CD4^+$ T cells and Treg cells (**B**).

Several studies have shown that IL-2/anti-IL-2 mAb complexes are potentially useful for immunotherapy. The use of the IL-2Sc resulted in a lower incidence of VLS in treated mice, compared to treatment with soluble IL-2 [206] and improved treatment outcomes when used for experimental cancer immunotherapy and for treatment against mouse viral and bacterial infections [207, 208]. Subsequently, the IL-2Jc has been shown to be efficacious in treating various mouse models of inflammation and autoimmune disease [209, 210]. Collectively, these studies showed that IL-2/anti-IL-2 mAb complexes selectively targeted

distinct T cells subsets and either enhanced or suppressed immune responses, depending on the cellular target.

The aim of work presented in this Chapter was to investigate the therapeutic potential of IL-2/anti-IL-2 mAb complexes for the treatment of visceral leishmaniasis using a mouse model.

5.2 **RESULTS**

5.2.1 The effects of IL-2/Anti-IL-2 mAb complex treatments during the chronic phase of *L. donovani* infection

I first tested whether IL-2/Anti-IL-2 mAb complexes could improve anti-parasitic immune responses during the chronic stage of *L. donovani* infection in the spleens of infected mice. C57BL/6 mice were infected with *L. donovani* and on days 28, 30 and 33 p.i., treated with IL-2Jc or IL-2Sc. Parasite burdens were measured on day 35 p.i. Control groups were given saline, recombinant IL-2 (rmIL-2), JES6.1A12 (JES6) or S4B6 mAbs.

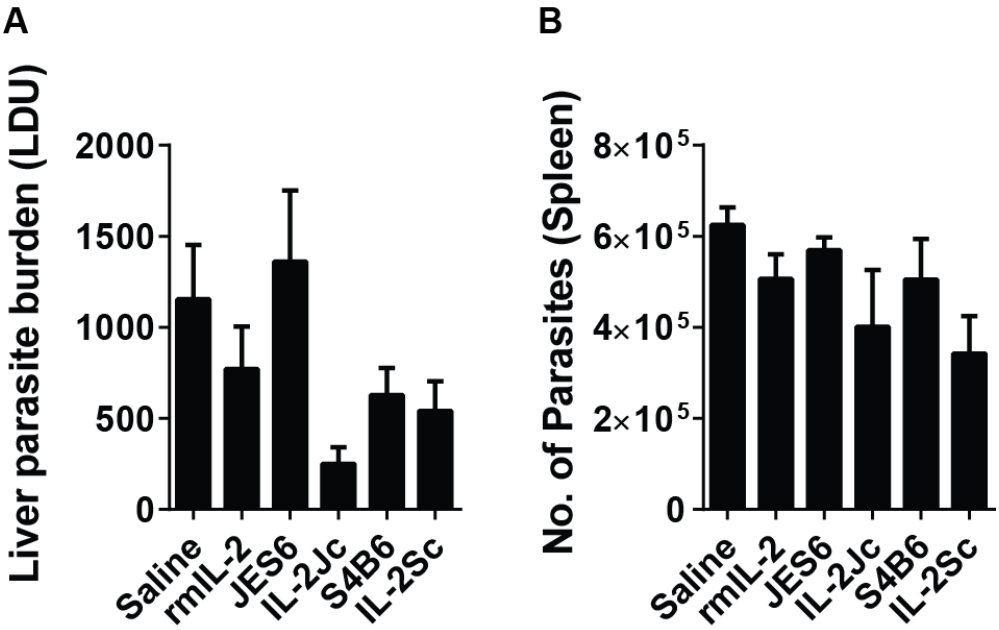

Figure 5.2: The effect of IL-2/Anti-IL-2 mAb complex treatment on the chronic phase of *L. donovani* infection.

Parasite burdens were determined in the livers (**A)** and spleens (**B**).of *L. donovani* infected mice treated with IL-2Sc or IL-2Jc on days 28, 30 and 33 p.i. *L. donovani* infected mice treated with saline were used as controls. Data are represented as the mean $^{+}$/- SEM at day 28 p.i. (n=5 mice per group). Results are representative of a single experiment.

Treatment with rmIL-2 or JES6 and S4B6 mAb alone no effect on parasite burdens, compared to the control group. Furthermore, I found no significant improvement in the control of parasite growth in the liver or spleen, compared to control groups (Figure 5.2A and B). Overall, IL-2/anti-IL-2 mAb complex treatment during the chronic stage of an *L. donovani* infection did not have a significant benefit on infection outcome, and I therefore turned my attention to testing the therapeutic potential of this treatment during the acute phase of infection.

5.2.2 Identification of immune cell populations expressing IL-2 receptors during an *L. donovani* infection

Prior to testing the effect of IL-2/anti-IL-2 mAb complex treatment on the acute phase of infection, I first examined the expression levels of IL-2Rs (CD25, CD122 and CD132), by FACS analysis on day 14 of an established *L. donovani* infection. Lymphocytes were isolated from organs of *L. donovani* infected mice and the frequency of NK cells, $CD4^+$ T cells, $CD8^+$ T cells and NK^- $TCR\beta^-$ cells expressing IL-2R was measured (Figure 5.3). Aged-matched, naïve (AMC) C57BL/6 mice were used as controls.

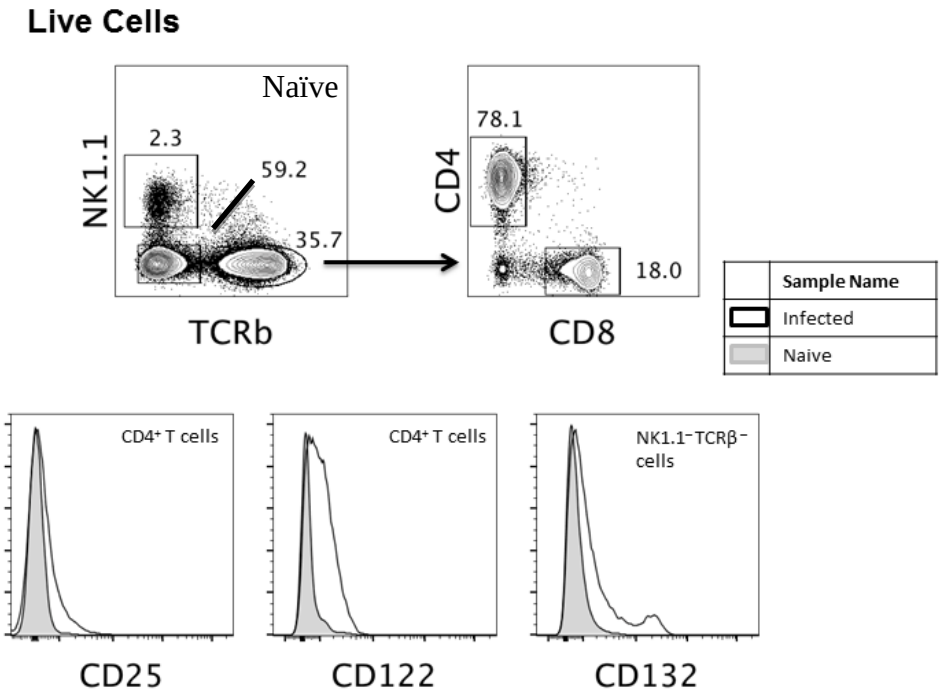

Figure 5.3: Representative gating strategies for the identification of IL-2 receptors on lymphocyte subsets in the liver.

Live cells were gated into $TCR\beta^-$ $NK\ 1.1^+$, $TCR\beta^-$ $NK\ 1.1^-$ and $TCR\beta^+$ $NK\ 1.1^-$ cell populations, and the $TCR\beta^+$ $NK\ 1.1^-$ cell population was then further gated, based on CD4 and CD8 expression. Cell surface staining was carried out on $TCR\beta^-$ $NK\ 1.1^+$, $TCR\beta^-$ $NK\ 1.1^-$, $CD4^+$ T cells and $CD8^+$ T cells to identify the frequency these cells expressing CD25, CD122 and CD132.

Immune cell analysis carried out on liver cells from mice infected with *L. donovani* for 14 days showed significant increases in the frequency of NK cell, $CD4^+$ and $CD8^+$ T cells expressing the IL-2Rs, compared to naïve mice (Figure 5.4). These results suggest that IL-2Rs are potential therapeutic targets during the acute phase of an *L. donovani* infection.

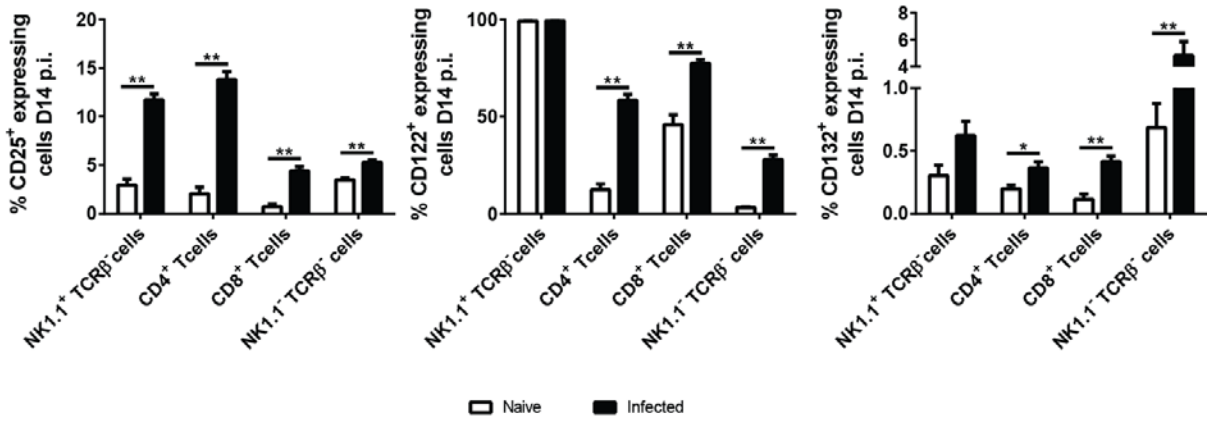

Figure 5.4: Expression of IL-2 receptors is enhanced during an *L. donovani* infection in the Liver.

Frequency of NK cells, $CD4^+$ and $CD8^+$ T cells expressing CD25, CD122 and CD132 was determined in the livers of *L. donovani* infected mice on day 14 p.i. Naïve C57BL/6 mice were used as AMC. Data is represented as the mean $^+$/- SEM at day 14 p.i., and statistical difference of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

5.2.3 Treatment with IL-2/Anti-IL-2 mAb complexes reduced *L. donovani* parasite burden

IL-2/Anti-IL-2 mAb complexes were administered either as a single dose (1x) on day 14 p.i. or as two doses (2x) on days 14 and 21 p.i. to establish the most effective dosing regimen. The measurement of parasite burdens and cellular analysis were carried out on day 28 p.i.

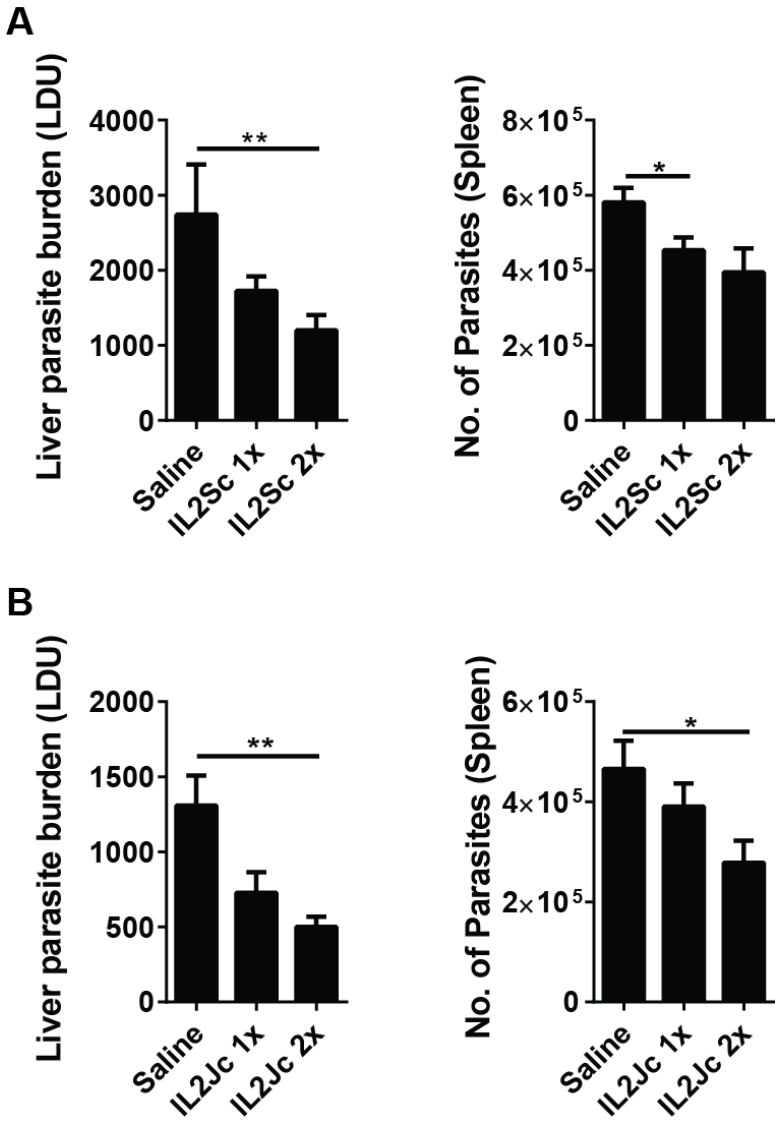

Figure 5.5: IL-2/Anti-IL-2 mAb complexes can improve control of *L. donovani* growth in the spleen and liver.

Parasite burdens were determined in the livers and spleens of *L. donovani* infected mice treated with one or two doses of IL-2Sc (**A**) or IL-2Jc (**B**). *L. donovani* infected mice treated with saline were used as controls. Data are represented as the mean $^{+}$/- SEM at day 28 p.i. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=10-15 mice per group). Results are representative of three different experiments.

Treatment with both the IL-2Sc and IL-2Jc resulted in a reduction in parasite burdens in both the liver and the spleen of infected animals (Figure 5.5A and B). There was a general trend for mice treated with two doses of IL-2Sc and IL-2Jc to control parasite burdens better than mice treated with a single dose. In addition, a significant reduction in the spleen was

only achieved with two doses of IL-2Jc. Therefore, for subsequent experiments, all treatments were carried out with two doses of IL-2/Anti-IL-2 mAb complexes.

5.2.4 Effect of IL-2/Anti-IL-2 mAb complex therapy on immune parameters during an *L. donovani* infection

As previously shown by others, IL-2Sc selectively enhances the expansion of NK cells and $CD8^+$ T cells, whereas the IL-2Jc selectively enhances the expansion of $CD4^+$ T cells and Treg cells [205]. Therefore, I wanted to determine if the control of parasite burden in the treated groups was due to the expansion of the above mentioned cell populations. To do this, I examined the frequency and number of activated $CD4^+$ T cells, $CD4^+$ $IFN\gamma^+$ T cells, Tr1 ($CD4^+$ $IFN\gamma^+$ $IL\text{-}10^+$) cells, Tregs ($CD4^+$ $Foxp3^+$ T cells), $CD8^+$ T cells, $CD8^+$ $IFN\gamma^+$ T cells, NK cells and NK $IFN\gamma^+$ cells in the different treatment groups (Figure 5.6 and Figure 5.8). This was performed as a comparative study between the IL-2Sc and IL-2Jc treatment groups, and both the livers and the spleens from infected mice were analysed.

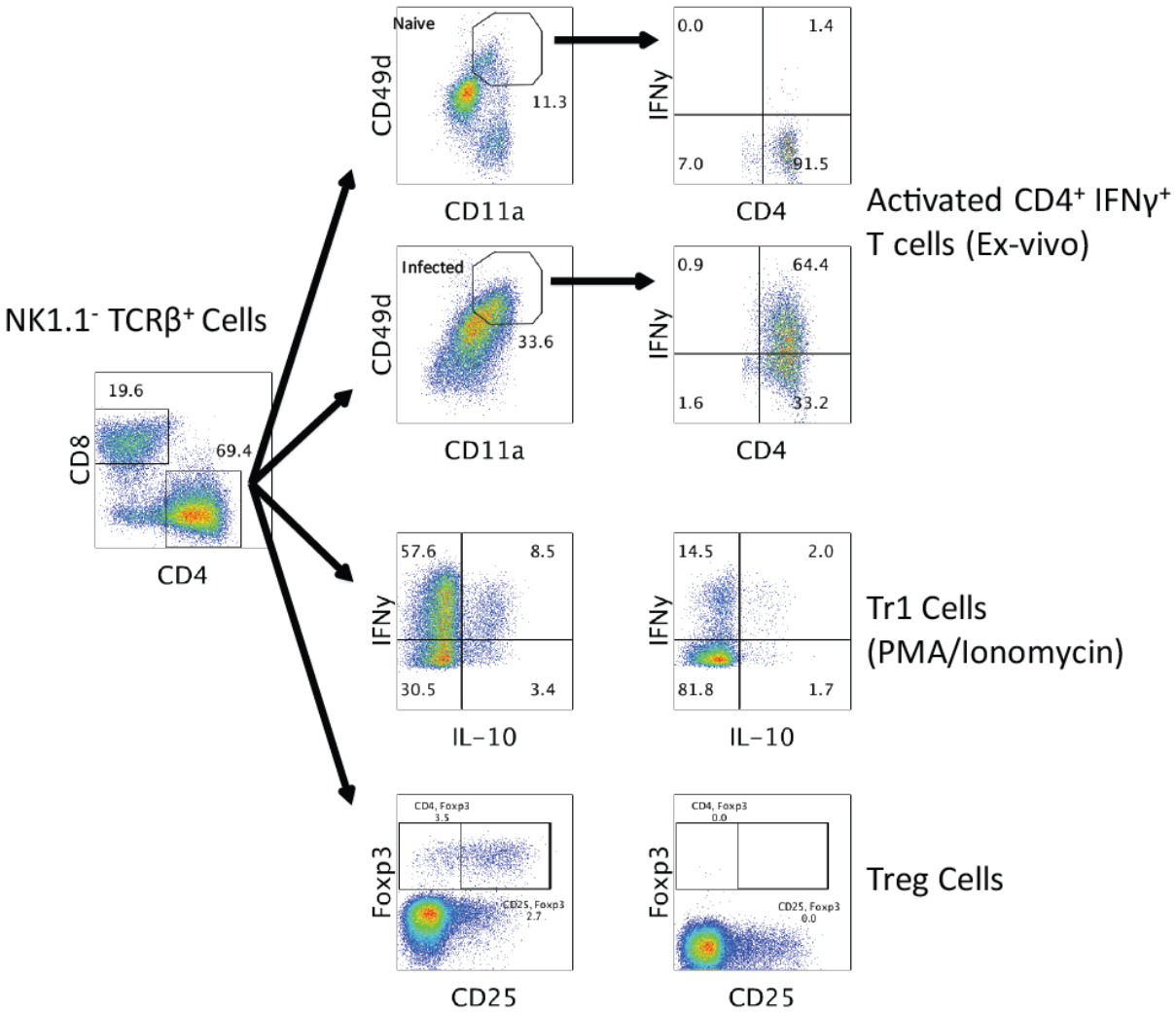

Figure 5.6: Representative sequential gating strategy for the isolation of immune cells.

From the NK 1.1$^-$ TCRβ$^+$ cell fraction, CD4$^+$ and CD8$^+$ T cells were gated. Activated CD4$^+$ T cells were selected based on CD49d and CD11a expression. Intracellular cytokine staining was carried out to identify IFNγ-producing CD4$^+$ T cells, Tr1 (IL-10- and IFNγ-producing CD4$^+$ T cells) cells in the activated cell fractions. Tregs (CD4$^+$ Foxp3$^+$) cells were identified, based on nuclear FoxP3 staining on the total CD4$^+$ T cell population.

Surprisingly, immune cell analysis carried out on liver and spleen cells showed only minor differences in the frequency of IFNγ-producing, activated CD4$^+$ T cells, Tr1 and Treg cells, between mice treated with two doses of either IL-2Jc or IL-2Sc, compared to control groups (Figure 5.7).

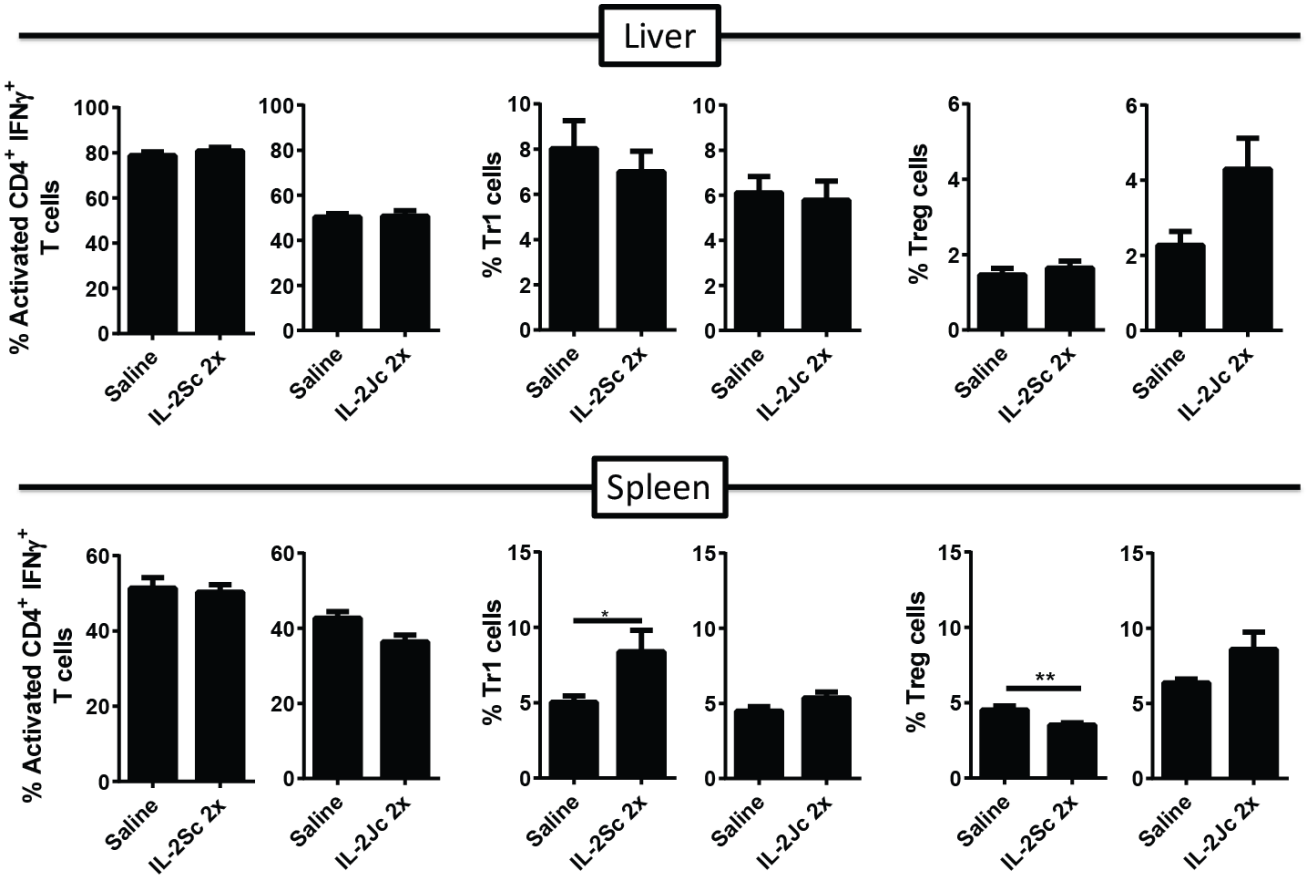

Figure 5.7: Treatment with IL-2Sc and IL-2Jc has little effect on the expansion of activated CD4$^+$ T cell expressing IFNγ, Tr1 and Treg cell population in the liver and spleen.

Hepatic and splenic Th1 (activated CD4$^+$ T cells expressing IFNγ), Tr1 and Treg cell frequencies were measured in mice infected with *L. donovani* and treated with IL-2Sc or IL-2Jc (separate experiments) on days 14 and 21 p.i. Mice given saline were used as controls. Data are represented as the mean $^+$/- SEM of T cell subset frequencies, as indicated, at day 28 p.i. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=10-11 mice per group). Results are representative of two different experiments.

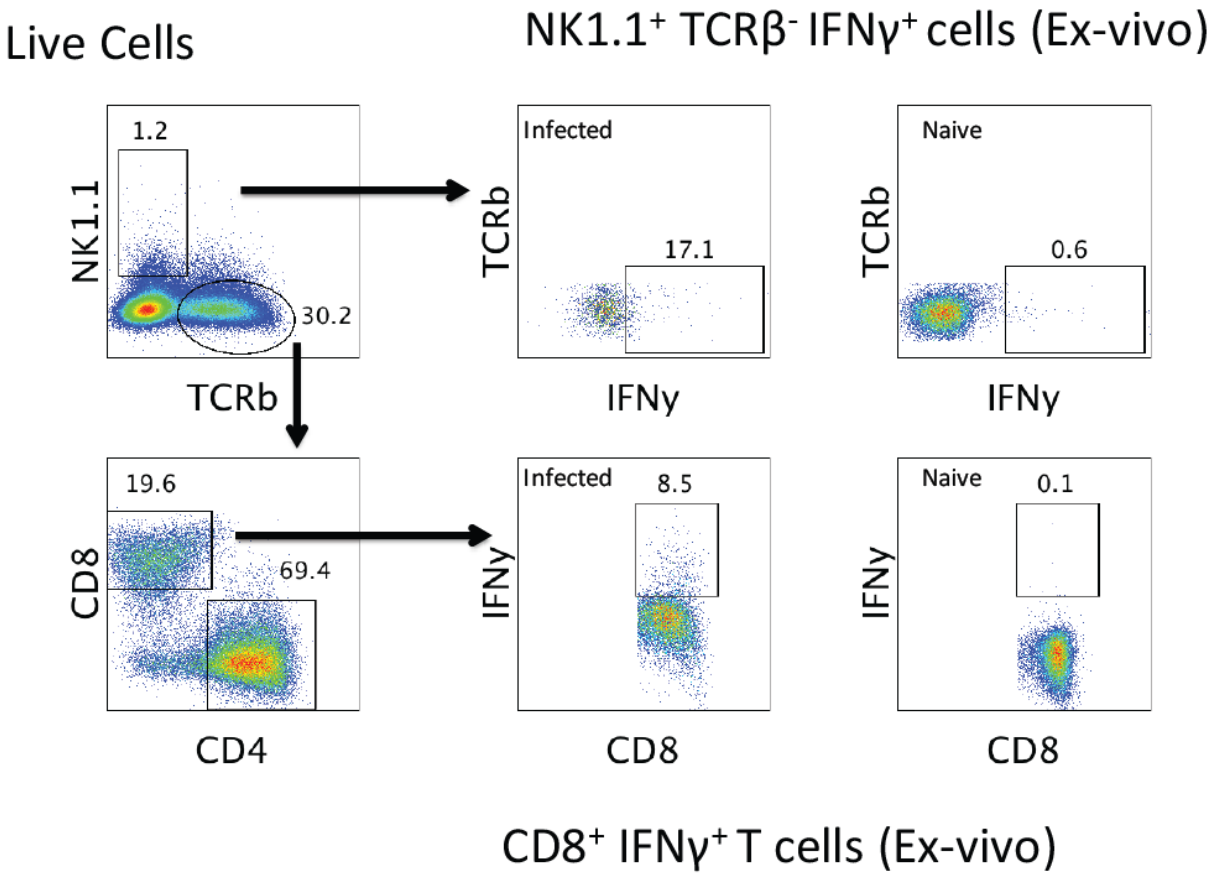

Figure 5.8: Representative gating strategy for the isolation of immune cells.

NK 1.1$^+$ TCRβ$^-$ cells, NK 1.1$^-$ TCRβ$^+$ cells, CD4$^+$ and CD8$^+$ T cells were gated from live cells. NK 1.1$^+$ TCRβ$^-$ cells and CD8$^+$ NK 1.1$^-$ TCRβ$^+$ cell were gated following intracellular IFNγ staining. The frequency of IFNγ-producing cells was then measured, as indicated.

Immune cell analysis carried out on liver and spleen cells showed only minor differences in the frequency of CD8$^+$ T cells and IFNγ-producing CD8$^+$ T cells between mice treated with two doses of either IL-2Jc or IL-2Sc, compared to control groups (Figure 5.9). Analysis of NK1.1$^+$ cells revealed a significant increase in the frequency of cells in the liver of IL-2Sc treated mice (Figure 5.10). Little effect of IL-2Sc or IL-2Jc treatment on IFNγ-producing NK1.1$^+$ cell was observed (Figure 5.10).

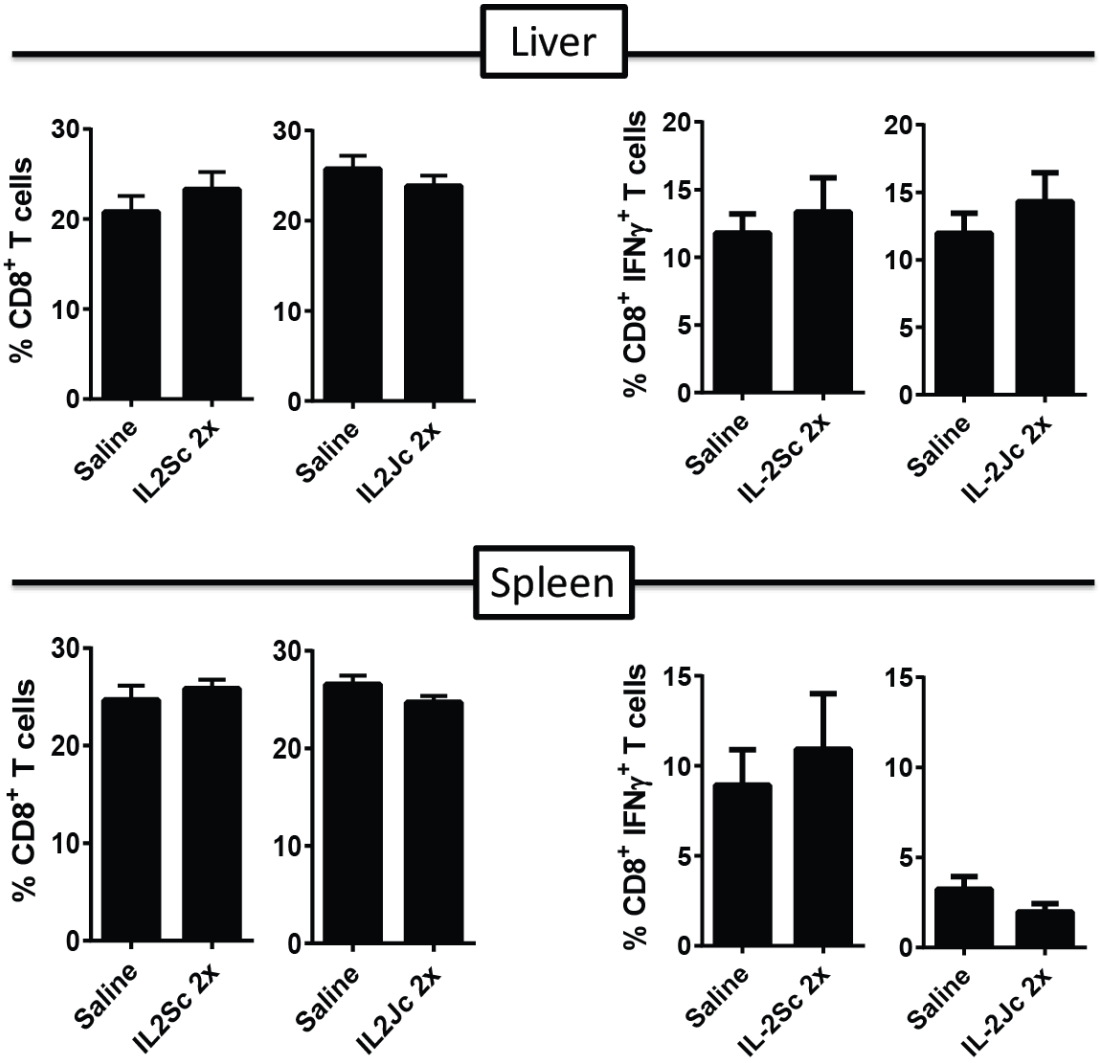

Figure 5.9: Treatment with IL-2Sc and IL-2Jc has little effect on the expansion of CD8$^+$ T cells in the liver or spleen.

Hepatic and splenic, CD8$^+$ and IFNγ expressing CD8$^+$ T cells were measured in mice infected with *L. donovani* and treated with either IL-2Sc or IL-2Jc (separate experiments) on days 14 and 21 p.i. Saline treated mice were used as controls. The frequency of CD8$^+$ and IFNγ expressing CD8$^+$ T cells are shown graphically. Data are represented as the mean $^+$/- SEM of CD8$^+$ T cells and IFNγ-expressing CD8$^+$ T cells at day 28 p.i. (n=10-11 mice per group). Results are representative of two different experiments.

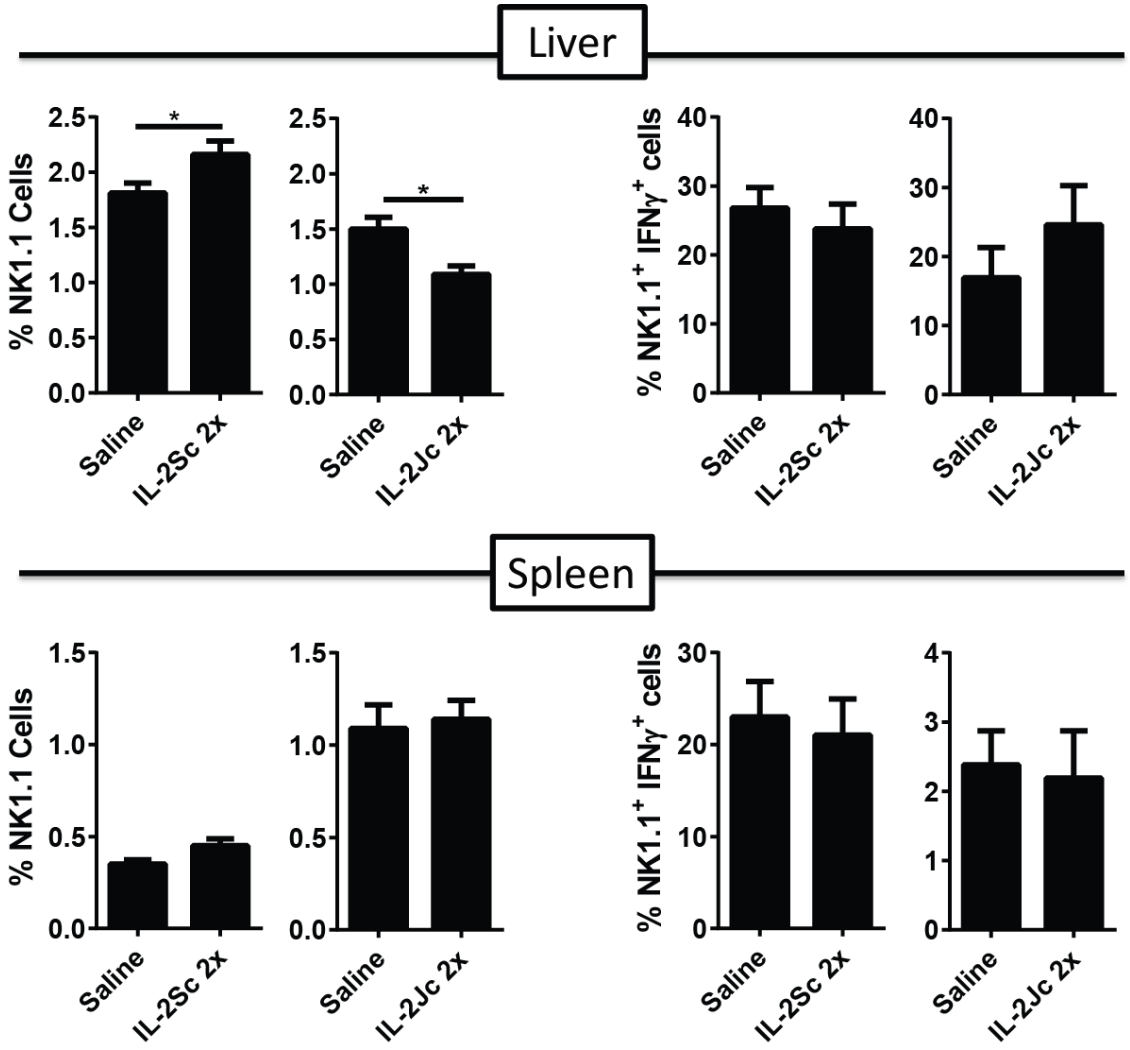

Figure 5.10: Treatment with IL-2Sc and IL-2Jc has little effect on the expansion of NK1.1 cells in the liver and spleen.

Hepatic and splenic, $NK1.1^+$ cells and IFNγ expressing $NK1.1^+$ cells were measured in mice infected with *L. donovani* treated with IL-2Sc or IL-2Jc (separate experiments) on days 14 and 21 p.i. Saline treated mice were used as controls. Data are represented as the mean $^+$/- SEM of the frequency of $NK1.1^+$ and IFNγ expressing $NK1.1^+$ cells at day 28 p.i., and statistical differences of $p < 0.05$ (*) are indicated (n=10-11 mice per group). Results are representative of two different experiments.

The above experiments indicated that two doses of IL-2/anti-IL-2 mAb complex treatment either with IL-2Sc or IL-2Jc resulted in significantly lower parasite burden, however, this protection was not associated with the cellular expansion of cells expressing the IL-2Rs, suggesting that other mechanisms of protective immunity may be involved.

5.2.5 Treg cells do not interfere with protection mediated by IL-2J complex treatment

As mentioned earlier, IL-2Jc has been reported to selectively expand cells expressing the high affinity heterotrimeric IL-2R expressed mainly on activated $CD4^+$ T cells and Treg cells. We know that conventional $CD4^+$ T cells responses are critical for anti-parasitic immunity against *L. donovani* [65], but that Treg cells may interfere with these cells [211, 212]. To test whether expansion of Treg cells impeded the effect of IL-2Jc on conventional $CD4^+$ T cells in *L. donovani* infected mice, Foxp3-GFP-DTR mice were used. These C57BL/6 mice contain a transgene encoding human diphtheria toxin receptor (DTR) inserted into the 3' untranslated region of Foxp3 [213]. The DTR is only found in the $Foxp3^+$ Treg cells of these mice, and injecting diphtheria toxin (DTx) intraperitoneally (i.p.), results in depletion of $Foxp3^+$ Tregs in these mice [213]. I hypothesised that eliminating Tregs in an established *L. donovani* infection, while treating with IL-2Jc, would further improve parasite control, as complex treatment would be targeted to activated $CD4^+$ T cells.

Foxp3-GFP-DTR mice were infected with *L. donovani* and treated with IL-2Jc on days 14 and 21 p.i. DTx treatment (8 ng/g i.p.) was started on day 12 p.i., and then every three days for the duration of the experiment. FACS analysis of Foxp3-GFP-DTR mice treated with DT indicated a reduction in Treg cell frequency (Figure 5.11A and B). The combination of IL-2Jc treatment and Treg cell depletion, however, did not further improve parasite burden in the treated animals, thus indicating that IL-2Jc anti-parasitic effects were not being inhibited by Treg cells (Figure 5.12A and B).

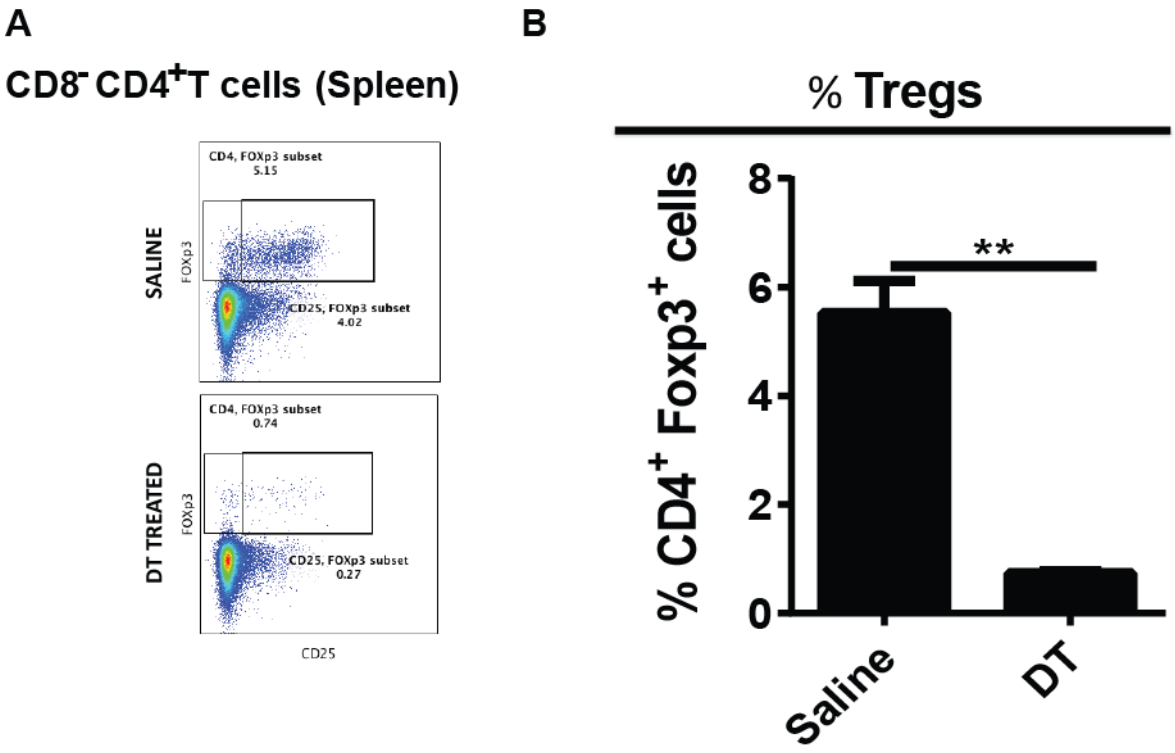

Figure 5.11: Foxp3-GFP-DTR mice treated with DT have a reduced frequency of Treg cells.

(**A**) Treg cells were identified by flow cytometry from the total $CD4^+$ T cells population. Foxp3 expression in $CD4^+$ spleen cells from *L. donovani* infected control Foxp3-GFP-DTR mice (Saline) and Foxp3-GFP-DTR mice treated with DT (started on day 12 p.i and then every three days for the duration of the experiment) was analysed. (**B**) Data are represented as the mean $^+$/- SEM of the frequency of hepatic and splenic Treg cells at day 28 p.i., and statistical differences of $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

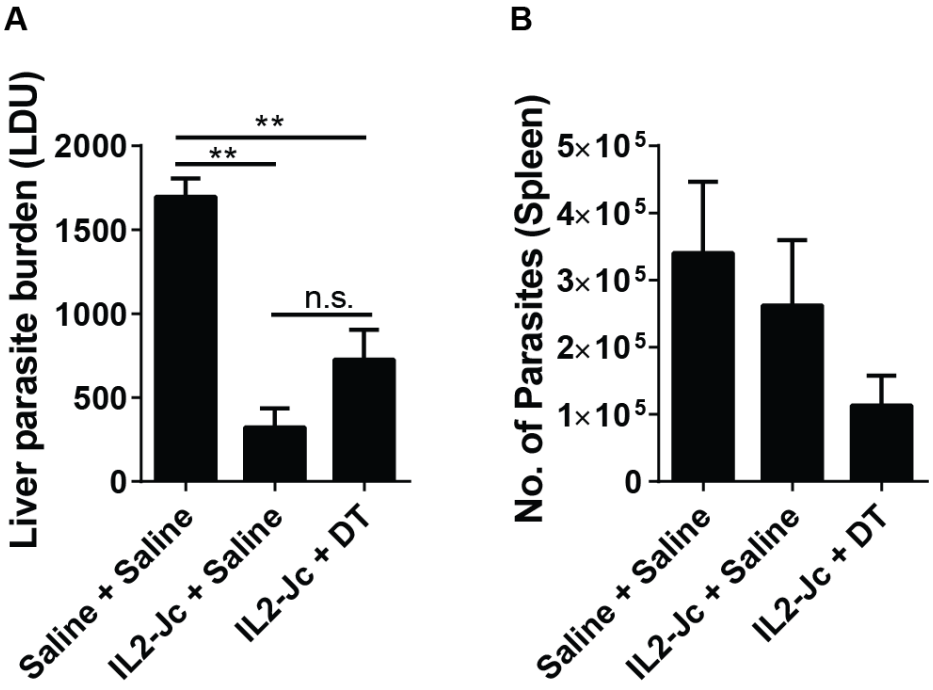

Figure 5.12: Tregs do not impair IL-2J complex-mediated protection.

Parasite burdens were determined in the livers (**A**) and spleens (**B**) of *L. donovani* infected Foxp3-GFP-DTR mice treated with two doses IL-2Jc. *L. donovani* infected mice treated with saline were used as control. DTx administration was started on day 12 p.i., and then every three days for the duration of the experiment. Data are represented as the mean $^{+}$/- SEM at day 28 p.i., and statistical differences of $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

Analysis of Treg cells from the livers and spleens of mice treated with IL-2Jc and DTx confirmed depletion of Foxp3^{+} T cells (Figure 5.13A and B). The depletion of Treg cells in IL-2Jc-treated mice did not change the frequency of Th1 and Tr1 cells in the spleen. However, there was a significantly lower frequency of Th1 and Tr1 cells in the liver following DT administration in IL-2Jc-treated mice, indicating different effects of Treg cell depletion in the spleen and liver after IL-2Jc treatment (Figure 5.13 A and B). Interestingly, although no difference in serum IFNγ and TNFα levels were observed in mice treated with IL-2Jc and depleted of Treg cells, IL-10 levels were increased, but this did not reach statistical significance (Figure 5.13 C).

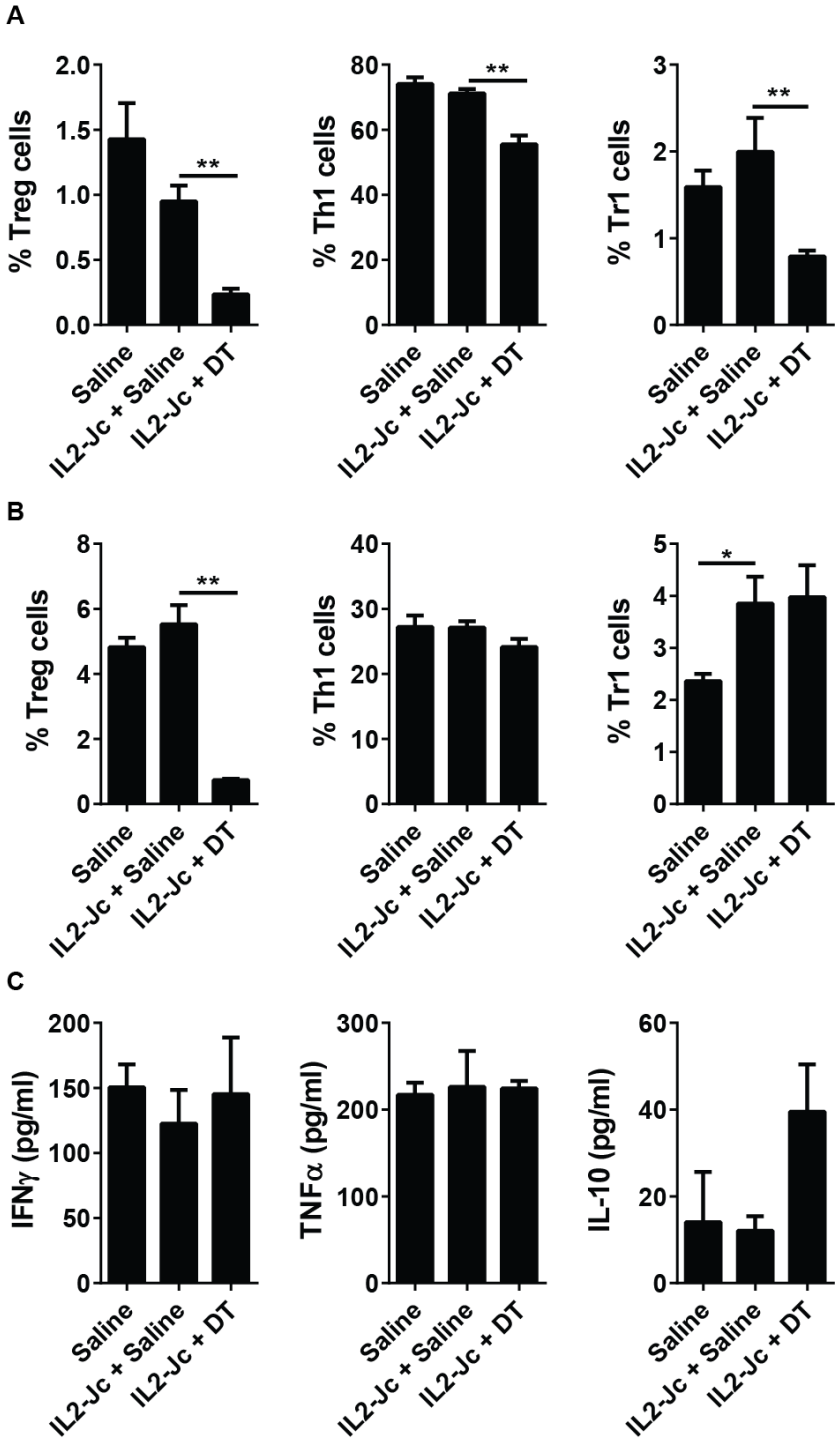

Figure 5.13: The impact of Treg cell depletion in IL-2J complex treated animals.

Hepatic (**A**) and splenic (**B**) Treg, Th1and Tr1 cellular responses were measured in *L. donovani* infected Foxp3-GFP-DTR mice, depleted of Tregs and treated with two doses of IL-2Jc. Saline was used as a control. The frequency of Treg, Th1 and Tr1 cells are shown graphically. (**C**) Serum IFNγ, TNFα and IL-10 levels were also measured in these mice. Data are represented as the mean $^{+}$/- SEM at day 28 p.i., and statistical differences of $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

5.2.6 The IL-2J complex mediates anti-parasitic effects in *L. donovani* -infected mice via $CD4^+$ T cells

I next tested the impact of depleting $CD4^+$ T cells in *L. donovani* infected mice treated with IL-2Jc. C57BL/6 mice were infected with *L. donovani* and treated with IL-2Jc on days 14 and 21 p.i. Anti-CD4 mAb (0.5 mg/mouse i.p.) treatment was started on day 12 p.i and then every three days for the duration of the experiment. FACS analysis of total $CD4^+$ T cells from infected mice confirmed depletion (Figure 5.14 A and B). Depletion of $CD4^+$ T cells in infected mice treated with IL-2Jc resulted in a significant increase in parasite burden compared to groups that were treated with an isotype control (ISO) mAb and IL-2Jc (Figure 5.15 A and B), thus indicating that $CD4^+$ T cells are required for IL-2Jc-mediated anti-parasitic immunity in experimental VL.

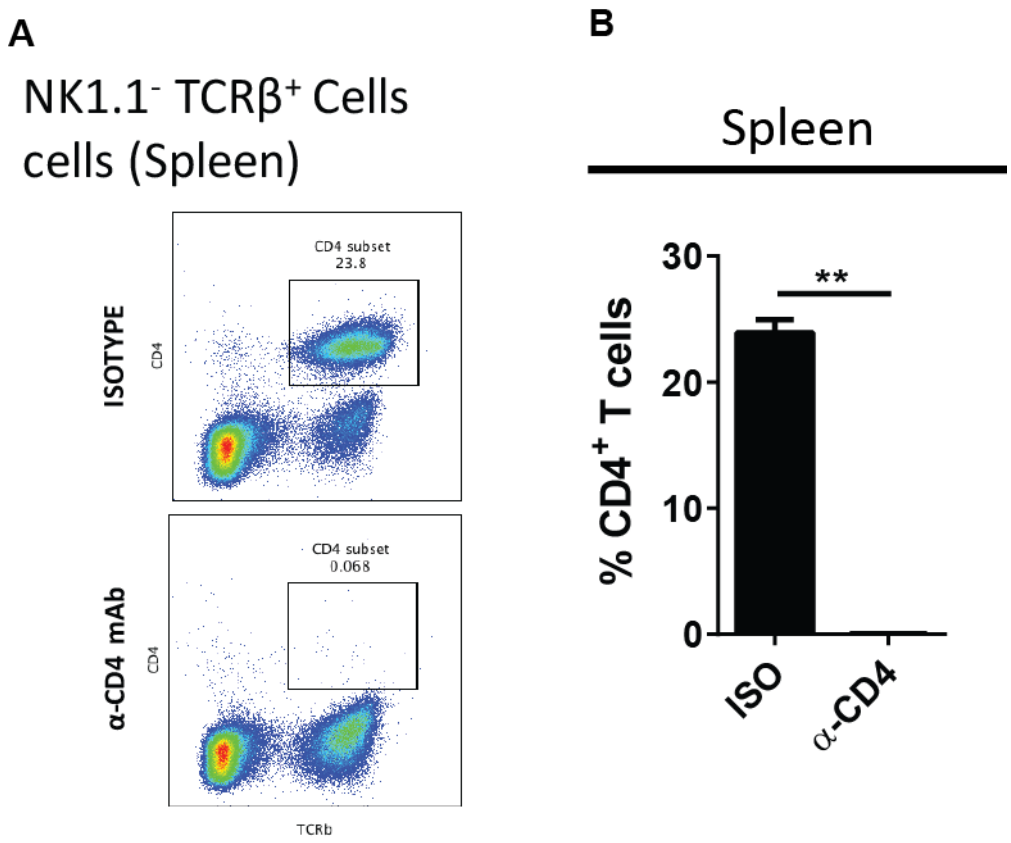

Figure 5.14: Administration of anti-CD4 mAb results in efficient $CD4^+$ T cell depletion.

(**A**) $CD4^+$ T cells were identified from $NK1.1^-$ $TCR\beta^+$ cells. $CD4^+$ T cells from spleens of *L. donovani* infected mice treated with α-CD4 mAb (started on day 12 p.i and then every three days for the duration of the experiment) were measured and compared to mice treated with an isotype control mAb (ISO)-. (B) The frequency of $CD4^+$ T cells in the spleen was significantly reduced, compared with control mice. Data are represented as the mean $^+$/- SEM at day 28 p.i., and statistical differences of $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

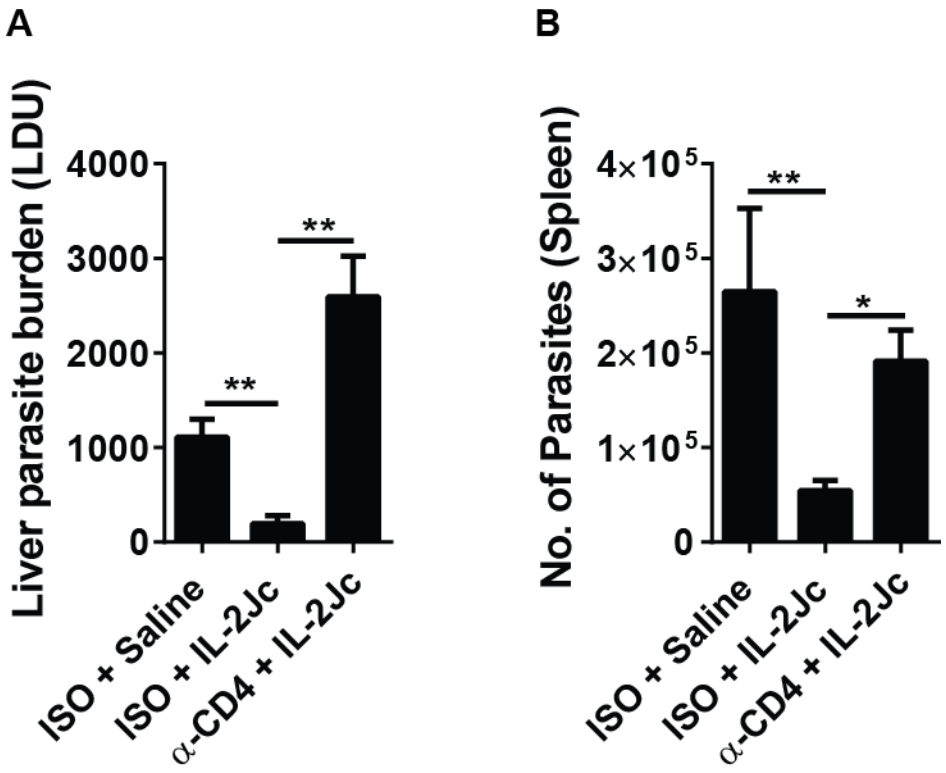

Figure 5.15: CD4$^+$ T cells are required for IL-2J complex mediated protection.

Parasite burdens were measured in the livers (**A**) and spleens (**B**) of *L. donovani* infected C57BL/6 mice treated with two doses of IL-2Jc. *L. donovani* infected mice treated with ISO were used as controls. Anti-CD4 mAb treatment commenced on day 12 p.i., and then every three days for the duration of the experiment. Data are represented as the mean $^+$/- SEM at day 28 p.i., and statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

Depletion of CD4$^+$ T cells in IL-2Jc-treated mice resulted in decreased CD4$^+$ T cell frequencies, as expected, but also significant increases in CD8$^+$ T cell and NK.1.1 cell numbers in the spleen, however when we looked at the number of CD8$^+$ T cell and NK.1.1 cell, there isn't a change due to the lack of CD4$^+$ T cells (Figure 5.16). These results indicated that in the presence of lower CD4$^+$ T cell, both CD8$^+$ T cells and NK cells can expand in response to IL-2Jc treatment. However, this expansion was not able to compensate for the loss of CD4$^+$ T cells in the control of parasite growth, highlighting the importance of CD4$^+$ T cells as anti-parasitic responder cells in mice treated with IL-2Jc.

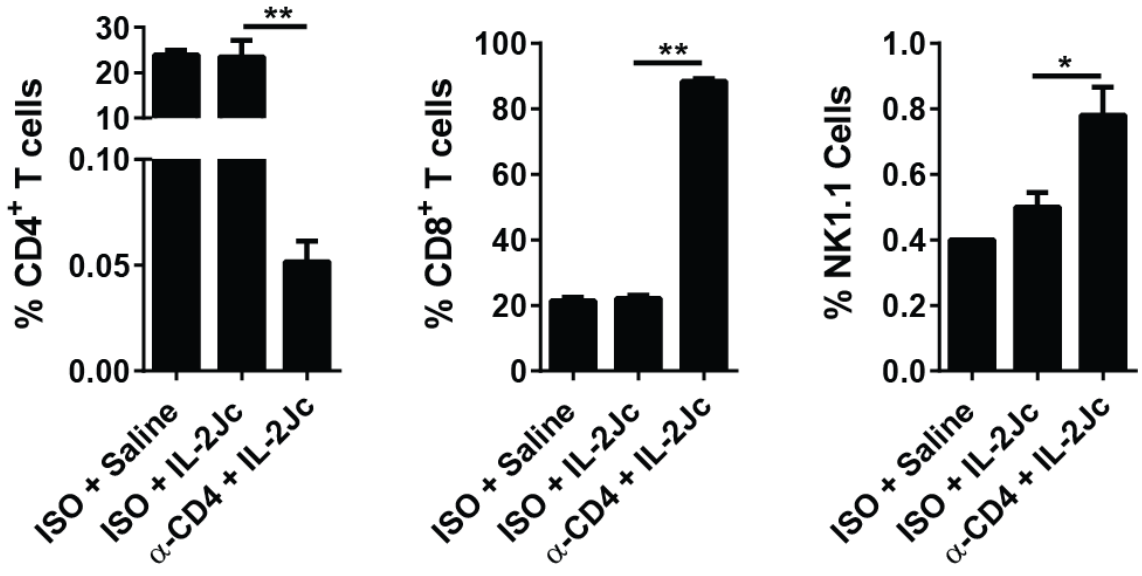

Figure 5.16: Depletion of CD4$^+$ T cells in IL-2J complex treated animals increased the frequency of CD8$^+$ T cell and NK1.1 cells.

Splenic CD4$^+$, CD8$^+$ T cell and NK1.1 cells were measured in *L. donovani* infected C57BL/6 mice depleted of CD4$^+$ T cells and treated with two doses of IL-2Jc. Isotype control mAb (ISO) treated mice were used as controls. Data are represented as the mean $^+$/- SEM of the frequency of CD4$^+$ T cells, CD8$^+$ T cells and NK1.1 cells at day 28 p.i. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

5.2.7 The IL-2S complex mediates anti-parasitic effects in *L. donovani* -infected mice via CD4$^+$ T cells, and not via CD8$^+$ T cells or NK cells

As mentioned earlier, IL-2Sc has been reported to selectively expand cells expressing the low affinity heterodimeric IL-2R found mainly on activated CD8$^+$ T cells and NK cells. We know that CD8$^+$ T cells aid in protection during with *L. donovani* [65], while studies have shown that NK cells are insufficient and not essential to control *L. donovani* [214, 215]. To test whether expansion of either CD8$^+$ T cells or NK cells enabled the effect of IL-2Sc on parasite burdens in *L. donovani* infected mice, I depleted each cell type. C57BL/6 mice were infected with *L. donovani* and treated with IL-2Sc on days 14 and 21 p.i. Anti-CD8β mAb

(0.5 mg/mouse i.p.) treatment was started on day 12 p.i and then every three days for the duration of the experiment. NK cell depletion was conducted using B6.NKp46Cre.iDTR mice [178]. DTx treatment (8 ng/g i.p.) was started on day 12 p.i., and then every three days for the duration of the experiment. FACS analysis of $CD8^+$ T cells or NK cells from treated mice confirmed 90% and 60% depletion, respectively. The combination of IL-2Sc treatment with depleting either $CD8^+$ T cells or NK cells did not alter the effects of IL-2Sc treatment on parasite burden in the treated animals, thus indicating that the anti-parasitic effects of IL-2Sc were not being mediated by either $CD8^+$ T cells or NK cells (Figure 5.17 A and B).

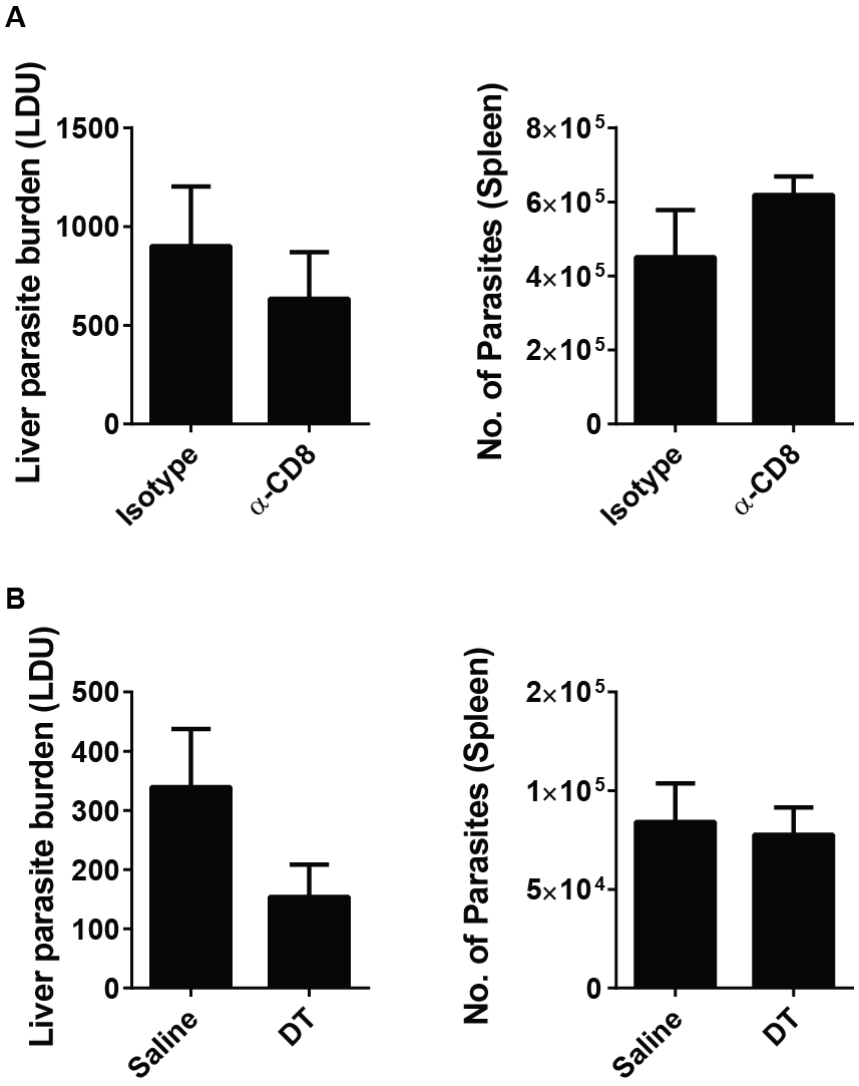

Figure 5.17: CD8$^+$ T cells and NK cells do not contribute to IL-2S complex-mediated protection.

Parasite burdens were determined in the livers and spleens of *L. donovani* infected C57BL/6 depleted of CD8$^+$ T cells (**A**) and B6.NKp46Cre.iDTR (**B**) mice treated with two doses of IL-2Sc. *L. donovani* infected mice treated with isotype control mAb or saline, respectively, were used as controls. Anti-CD8β mAb and DTx administration was commenced on day 12 p.i., and then every three days for the duration of the experiment. Data are represented as the mean $^+$/- SEM at day 28 p.i., (n=5 mice per group). Results are representative of a single experiment.

Since conventional CD4$^+$ T cells can also express the low affinity heterodimeric IL-2R, I next tested the impact of depleting CD4$^+$ T cells in *L. donovani* infected mice treated with IL-2Sc. C57BL/6 mice were infected with *L. donovani* and treated with IL-2Sc on days 14 and 21 p.i. Anti-CD4 mAb (0.5 mg/mouse i.p.) treatment was started on day 12 p.i., and then every three days for the duration of the experiment. FACS analysis of total CD4$^+$ T cells from

infected mice confirmed greater than 98% depletion (Figure 5.14 A and B). Depletion of $CD4^+$ T cells in infected mice treated with IL-2Sc resulted in a significant increase in parasite burden, compared to groups that were treated with an isotype control (ISO) mAb and IL-2Sc (Figure 5.18 A and B), thus indicating that $CD4^+$ T cells were required for IL-2Sc-mediated anti-parasitic immunity in experimental VL.

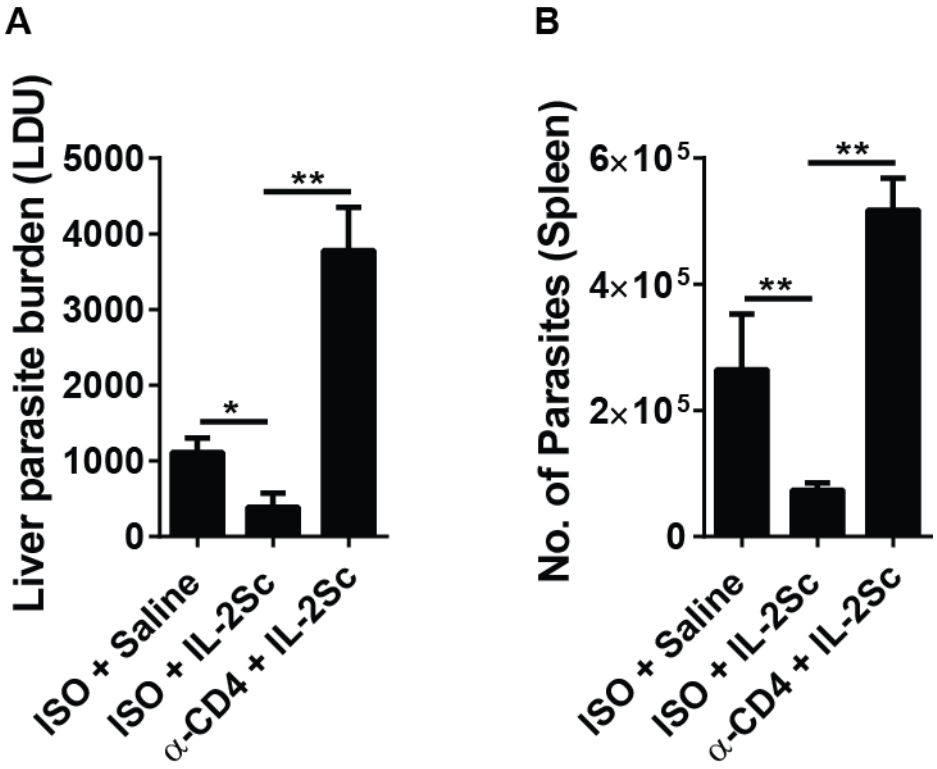

Figure 5.18: $CD4^+$ T cells are required for IL-2S complex mediated protection.

Parasite burdens were measured in the livers (**A**) and spleens (**B**) of *L. donovani* infected C57BL/6 mice treated with two doses of IL-2Sc. *L. donovani* infected mice treated with an isotype control mAb (ISO) were used as controls. Anti-CD4 mAb treatment commenced on day 12 p.i., and then every three days for the duration of the experiment. Data are represented as the mean $^+$/- SEM at day 28 p.i., and statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

5.3 **DISCUSSION**

In this chapter the potential of IL-2 therapy using IL-2/Anti-IL-2 mAb complexes to improve experimental VL disease outcome was investigated. To achieve this, two commonly used IL-2/anti-IL-2 mAb complexes, the IL-2J complex comprising rmIL-2/JES6.1A12 mAb and the IL-2S complex comprising of rmIL-2/S4B6 mAb, were used. Previous experiments have shown that treatment with exogenous IL-2 improved anti-parasitic immune responses against VL [200], and several other studies have shown that IL-2/anti-IL-2 complexes selectively target different T cell subsets to either enhance or suppress immune responses in different disease settings [207-210]. Hence, to investigate the effect of IL-2/anti-IL-2 mAb complex therapy during VL, *L. donovani* infected mice were treated with either IL-2Jc or the IL-2Sc.

In VL, as well as in EVL, the spleen is a site of persistent and chronic infection. Initially, I tried to use IL-2/anti-IL-2 mAb complex therapy to target this organ to improve anti-parasitic immune responses during EVL. Treatment commenced on day 28 p.i., when a chronic infection had established in the spleen [73, 75]. However, I found little indication that IL-2/anti-IL-2 mAb complexes had any significant effect on parasite burdens. Overall, the result indicated that the chronic stage of the infection wasn't the ideal setting to assess the therapeutic effects of these complexes. Therefore, I turned my attention to the effect of IL-2/anti-IL-2 mAb complex therapy during the acute phase of the infection and found that mice responded better to IL-2/anti-IL-2 mAb complex therapy given in the early stages of the infection (from day 14 p.i.), where two doses of either IL-2Jc or IL-2Sc was better at controlling parasite burdens than a single dose. Analysis of immune cell populations

associated with IL-2Jc or IL-2c treatment, however, failed to reveal any significant changes that might account for the protective immunity initiated by this treatment.

Earlier experiments have shown that in an *L. donovani* infection, Treg cells inhibit protective immune responses and the cellular recruitment required for parasite control [211, 216]. Therefore, one potential adverse effect of IL-2Jc treatment was the expansion and/or activation of Treg cells with detrimental effects on anti-parasitic T cell responses. However, when Treg cells were depleted by DT administration in Foxp3-GFP-DTR treated with IL-2Jc, there was no significant improvement in the anti-parasitic effects of IL-2Jc, indicating that this complex was not promoting Treg cell expansion of activation. To test if the IL-2Jc mediated protection involved effects on conventional $CD4^+$ T cells, anti-CD4 mAb was used to deplete these cells during the IL-2Jc treatment period. This resulted in disease exacerbation. A similar effect was observed when $CD4^+$ T cells were depleted during the IL-2Sc treatment period, while depletion of $CD8^+$ T cells and NK cells had no effect on parasite burdens of IL-2Sc treated mice. Thus, these results show the requirement of conventional $CD4^+$ T cells for IL-2Jc and IL-2Sc mediated protection in experimental VL.

IL-2 is a pleiotropic cytokine responsible for the development and maintenance of Treg cells [217], expansion of Th1 [218], Th2 [219] and Th9 [220] cells, while suppressing $CD4^+$ T cell differentiation into Th17 cells [221]. Stimulation of NK cells and $CD8^+$ T cells by IL-2 results in the proliferation, increased cytotoxic activity and cytokine production [199]. Recent studies have shown that type 2 innate lymphoid cells (ILC2) express high levels of CD25 and can proliferate in reposes to low-dose IL-2 through activation of the trimeric IL-2R [222]. This supplements the production of IL-5, which expands eosinophil

populations, resulting in eosinophilia [223]. Hence, alteration to IL-2 levels has the potential to cause unexpected consequences, and binding of IL-2 to the IL-2Rs might lead to unexpected changes *in-vivo*. In my experiments, treatment with IL-2Sc did not result in significant expansion of NK cells or $CD8^+$ T cells. Similarly, treatment with IL-2Jc did not cause significant expansion of Treg cells or activated $CD4^+$ T cells. One possible explanation for this result is that expansion of these cell populations occurred rapidly post-treatment and waned by the time cellular responses were measured (7 days later). However, the fact that both IL-2Jc and IL-2Sc treatment resulted in significant reductions in parasite burden, indicates that IL-2/anti-IL-2 mAb complexes stimulated potent anti-parasitic immunity.

In addition to IL-2/anti-IL2 mAb complexes, other IL-2-related treatments are currently being investigated. Early studies with IL-2 fused to carrier proteins, such as the Fc domains of IgG, improved cytokine half-life [224]. More recently, an increasing number of studies in animals and humans have shown that multiple low doses of IL-2 have fewer adverse effects and favourable immune outcomes [225, 226]. Several mutant forms of IL-2 have also been developed with transformed binding sites that change interactions between the cytokine and IL-2Rs, resulting in altered function. Shanafelt, *et al*., have reported the development of a mutant form of IL-2 known as BAY 50-4798. This molecule has a higher affinity for trimeric IL-2Rαβγ, relative to dimeric IL-2Rβγ, and promoted enhanced T cell activation, without any effects on NK 1.1. cells [227]. Studies using IL-2 and with various check-point inhibitors have also shown promising results for immunotherapy for cancer [228, 229]. Together with my results, these studies demonstrate the potential of IL-2 therapy in targeting specific immune parameters to improve disease outcomes. However, targeting the right immune cells in the host to optimise effects and understanding the mechanism of protective immunity remains a challenge.

In this Chapter, I showed that IL-2/anti-IL2 mAb complex therapy with either IL-2Jc or IL-2Sc significantly reduced parasite burdens in experimental VL. However, surprisingly, I observed no expansion of potential target cell populations and the mechanism of enhanced parasite control remains unclear. Overall, IL-2 therapy warrants further investigation for development as a treatment for clinical VL.

Chapter 6: To compare different methods of parasite attenuation and establish whether a live, attenuated, whole parasite vaccine can protect against experimental VL

6.1 INTRODUCTION

The most common VL treatment for the last 60 years has been antimonial chemotherapy [230]. Pentavalent antimonials, such as sodium stibogluconate, pentostam, meglumine antimonite and glucantime, have been the mainstay of antimonial therapy [231]. However, there is now considerable parasite resistance against these drugs, especially in North-Eastern India and surrounding areas [232]. Therefore, although these drugs are still employed to treat VL in Africa, drugs such as Amphotericin B, Miltefosine, aminosidine (paromomycin) and sitamaquine have been developed as effective treatments against VL in areas of antimonial drug resistance [232]. However, these drugs are still far from ideal because of cost, toxicity, development of parasite drug resistance after prolonged use and duration of treatment times [4]. Some progress has been made recently in addressing this latter issue [164], where a single dose of lipid formulation of Amphotericin B (Ambisome), was effective in treating VL patients with lower toxicity outcomes compared to the conventional drug treatment. However, there are still concerns that this single dose of treatment may eventually result in the development of drug-resistant parasites. To further address this concern of parasite drug resistance, combination drug therapy is being developed [158, 233]. However, studies in experimental VL suggested that *L. donovani* can develop drug resistance, even to drug combination therapy [234]. Despite the arsenal of anti-

protozoan drugs available, it is unlikely that chemotherapy alone will control and eradicate the disease, and thus, there is an urgent need for an effective and protective vaccine against VL.

The development of a vaccine to prevent leishmaniasis has been a long-term goal for researchers in the field. At present there are no licensed vaccines that prevent leishmaniasis [235]. In theory, a vaccine to prevent leishmaniasis should be possible, as indicated by past programs of "leishmanisation". This process involves the deliberate infection of people with CL-causing parasite species on unexposed areas of the body to establish an infection that is controlled in most individuals, resulting in long-term protection [236]. This technique was practised for centuries throughout the Middle East and parts of Asia, and large-scale trials were carried out in the former Soviet Union and Israel with some success [171, 172] but this relied on parasites that were viable and infective [173]. However, despite the solid immunity that develops in most individuals, this approach has largely been abandoned due to complications in some individuals including large skin lesions, exacerbation of skin diseases and poor responses to the vaccine [7, 8]. The vaccines currently being developed against VL can be divided into three groups; first generation vaccines which involve vaccination with live-attenuated or killed parasite; second generation vaccines which involve genetically modified parasites, subunit vaccines or recombinant virus and bacteria recombinant parasite protein expression systems; and third generation vaccines which consist of plasmid DNA and viral based vaccines encoding virulent *Leishmania* proteins [43, 235, 237]. However killed parasite, recombinant DNA and protein vaccine appear to be ineffective due to the short-term immunity they induce [238]. Whole irradiated or biochemically altered parasites have been shown to confer potent protection in an experimental model of VL [239, 240], similar to the

results achieved by leishmanisation. Hence, for a robust protective immune response against VL, whole parasite vaccines show promise.

Various mouse models of *Leishmania* infection have shown that resistance to the parasite is mediated by cellular immune responses, primarily involving Th1 responses, which are IL-12 dependent [241]. IFNγ and TNFα produced by parasite-specific Th1 cells drive the effector function of macrophages to generate reactive oxygen and nitrogen species required for intracellular parasite killing by macrophages [87]. IL-10, an immune regulatory cytokine produce by parasite-specific $CD4^+$ T cells, plays an active role in supressing anti-parasitic Th1 immune responses and promotes parasite persistence in VL patients [54]. Thus, Th1 responses are important for the elimination of parasites in infected individuals. Understanding the requirements and conditions required for the generation of protective Th1 responses against *Leishmania* parasite is important for developing an effective vaccine.

The previous chapters focused on promoting $CD4^+$ T cell responses during established experimental VL via immune modulatory techniques such as combination immune treatment and chemotherapy, as well as IL-2 complex therapy. These experiments showed us that sub-optimal $CD4^+$ T cell responses generated during VL can be improved given the right immune conditions. In this chapter, I aimed to develop a whole parasite vaccine to generate protective parasite-specific $CD4^+$ T cell responses against VL.

6.2 **RESULTS**

6.2.1 The effects of immunization with irradiated whole *L. donovani* promastigotes

My first vaccine study was carried out to identify the vaccine regime best suited to generate a potent immune response. I used *L. donovani* promastigotes as my parasite vaccine for immunization as this form of the parasite is transmitted to the host when the sand-fly takes its blood meal [242]. Also, *L. donovani* promastigotes are easy to culture *in-vitro* [243], and large quantities of the parasites can be produced. The vaccine regime followed was previously described by Scott *et al.* [243]. Briefly, C57BL/6 mice were immunized with four doses of irradiated *L. donovani* promastigotes given either intravenously (Irr LV9 i.v.) or intraperitoneally (Irr LV9 i.p.) and challenged with live *L. donovani* amastigotes two weeks after the last immunization and sacrificed on day 14 and day 28 post challenge (p.c.) to assess hepatic and splenic parasite burdens (Figure 6.1.1). Naïve age matched mice were used as controls (AMC), and these were infected with *L. donovani* at the same time as the immunized mice were challenged.

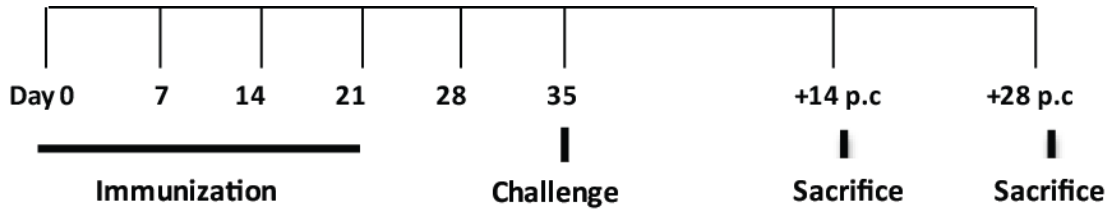

Figure 6.1.1: Experimental timeline for assessment of protection against *L. donovani*.

C57BL/6 mice were immunized with *L. donovani* promastigotes irradiated at 500 Gys ($2x10^7$ parasites i.v. or $5x10^7$ parasites i.p.). Primary immunization was carried out on day 0 and subsequent boost on days 7, 14, and 21. Two weeks after the final booster (i.e., day 35), mice were challenged with 2 $x10^7$ live *L. donovani* amastigotes. Mice were sacrificed on days 14 and day 28 p.c. Naïve AMC were infected with *L. donovani* on day 35 in the above scheme.

Analysis of hepatic and splenic parasite burdens showed no improvement in parasite control on either day 14 or day 28 p.c., between the immunized and AMC groups (Figure 6.2 A and B). These results indicate that this immunization regime used was not effective.

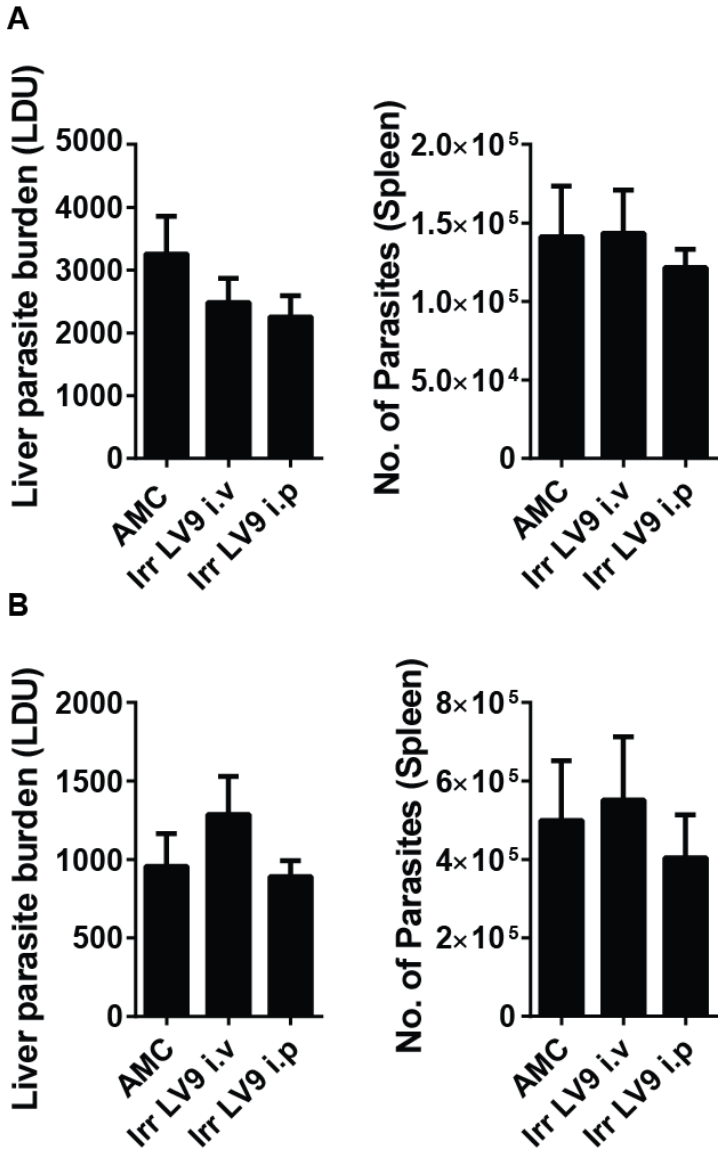

Figure 6.2: Failure of previously reported immunisation regime to protect against VL.

Parasite burdens were determined in livers and spleens of *L. donovani* challenged mice on day 14 (**A**) and day 28 p.c. (**B**). Prior to challenge mice were immunized with irradiated (500 Gys) *L. donovani* promastigotes ($2x10^7$ parasites i.v. or $5x10^7$ parasites i.p.). Aged matched mice were used as controls (AMC). Data are presented as the mean +/- SEM at day 14 and day 28 p.i. (n=5 mice per group). Results are representative of a single experiment.

6.2.2 The effect of immunization with either radio- or chemically-attenuated whole *L. donovani* amastigotes

Given the above results, I next investigated the effect of immunization with attenuated amastigotes instead of promastigotes. This was done under the rationale that the challenge infection was initiated with amastigotes, and so the immune cells should be primed for amastigote antigens. In addition to using irradiated amastigotes, I wanted to assess the potential of using chemically-attenuated parasites for vaccination. The chemical used to attenuate the *L. donovani* parasites was TH-III-149 (tafuramycin A: TFA), which is an alkylating agent that irreversibly alters the parasites DNA at multiple sites preventing proliferation, but leaves the parasite viable [244]. Furthermore, to assess if multiple immunization doses improved the control of parasite burden in vaccinated mice, C57BL/6 mice were immunized i.v. with either a single (1x) or two doses (2x) of irradiated (Irr LV9) or chemically-attenuated (TFA LV9) *L. donovani* amastigotes (Figure 6.1.2). The interval between the primary immunization and subsequent boost was increased to four weeks instead of seven days, based on reports that longer intervals between vaccine administrations can improve efficacy [245]. Mice were challenged with live *L. donovani* amastigote four weeks after the final immunization (day 56), and they were sacrificed on day 14 p.c. Naïve AMC were infected with *L. donovani* at the same time as the immunized mice were challenged.

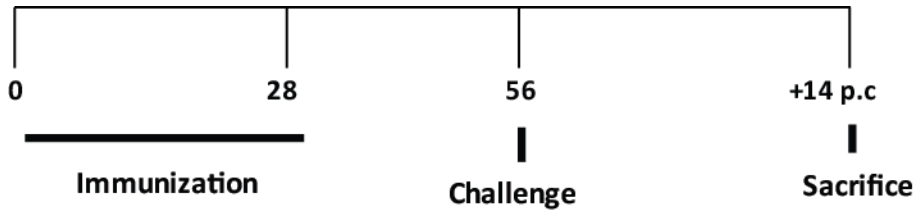

Figure 6.1.2: Experimental timeline for assessment of protection against *L. donovani*.

C57BL/6 mice were immunized with *L. donovani* amastigotes irradiated at 500 Gys or chemically-attenuated with TFA ($2x10^7$ parasites i.v.). For mice receiving two immunizations (2x), the primary dose was given on day 0 and subsequent boost on day 28. Some mice received a single immunization on day 28 (1x). Four weeks post final immunization i.e. day 56 mice were challenged with 2 $x10^7$ live *L. donovani* amastigotes and were sacrificed on day 14 p.c.

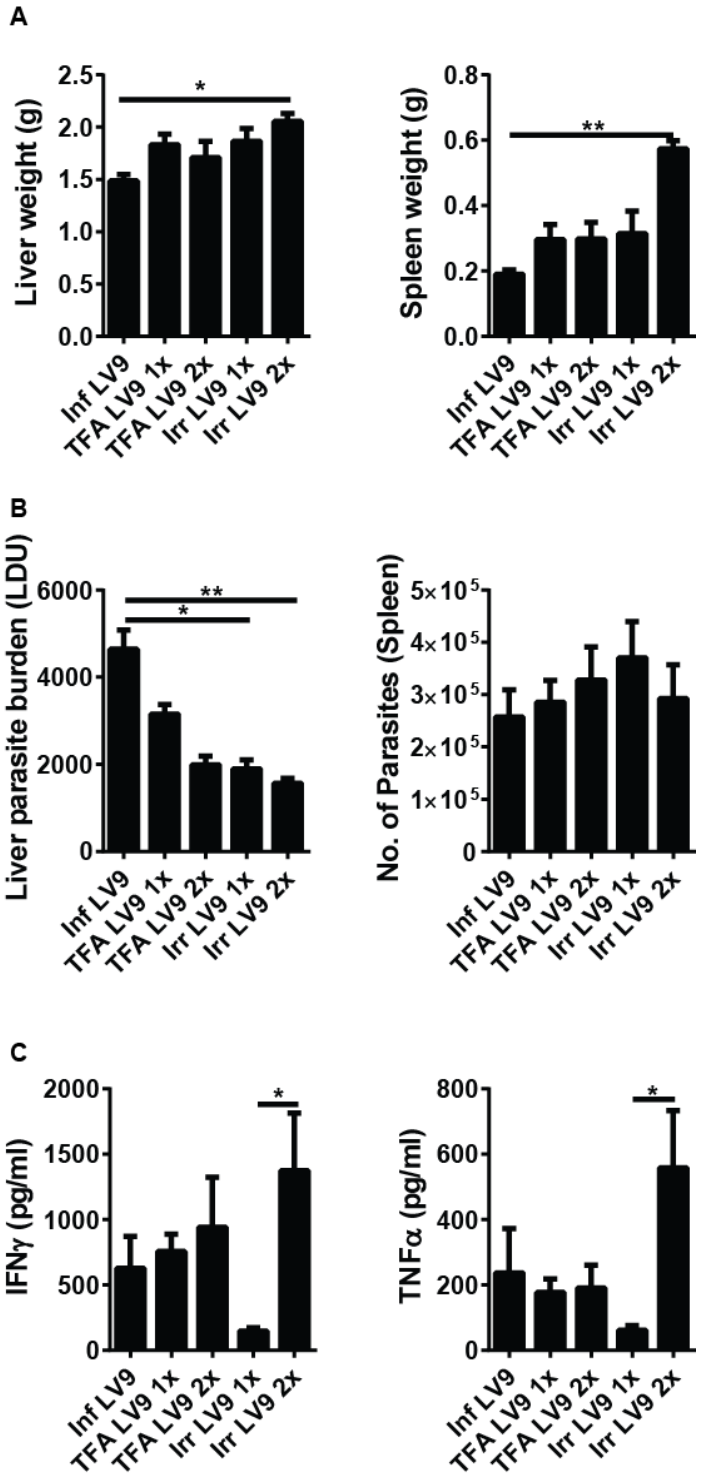

Figure 6.3: Immunization with irradiated *L. donovani* amastigotes improves parasite control in the liver.

Organ weight (**A**) and parasite burdens (**B**) were determined in the livers and spleens of mice immunized with Irr LV9 or TFA LV9 (1x or 2x), as indicated. Mice were challenged with *L. donovani* four weeks after the last immunization and sacrificed on day 14 p.c. AMC were infected with *L. donovani* on the day of challenge. (**C**) IFNγ and TNFα (pg/ml) in the serum of infected and vaccinated mice. Data are presented as the mean +/- SEM at day 14 p.c. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

Mice immunized with two doses of irradiated *L. donovani* amastigotes (Irr LV9) showed a significant increase in liver and spleen weights (Figure 6.3 A), indicative of increased immune cell infiltration into these organs [96]. Assessment of liver parasite burdens showed a significant decrease in parasite burden in mice immunized with either a single or two doses of Irr LV9, while mice immunized with two doses of chemically-attenuated parasite (TFA LV9) appeared to be better protected compared to mice immunized with a single dose, although not statistically different. (Figure 6.3 B). In the spleen, however, no change in parasite burden was observed in the immunized groups compared to the AMC group. Serum cytokine analysis revealed elevated levels of pro-inflammatory cytokines IFNγ and TNFα in groups immunized with two doses of Irr LV9 (Figure 6.3 C).

These results indicated that immunization with irradiated LV9 provided better protection in the liver than immunization with chemically-attenuated parasites. Moreover, two immunizations with irradiated LV9 resulted in enhanced levels of pro-inflammatory cytokines over the single immunization groups, as indicated by elevated levels of IFNγ and TNFα.

I next examined the frequency of Th1 ($Tbet^+$ IFNγ-producing $CD4^+$ T cells) cells and terminally differentiated Th1 ($Tbet^+$ $KLRG\text{-}1^+$ $CD4^+$ T cells) cells in the different immunized groups (please refer to previous chapters for representative gating strategy).

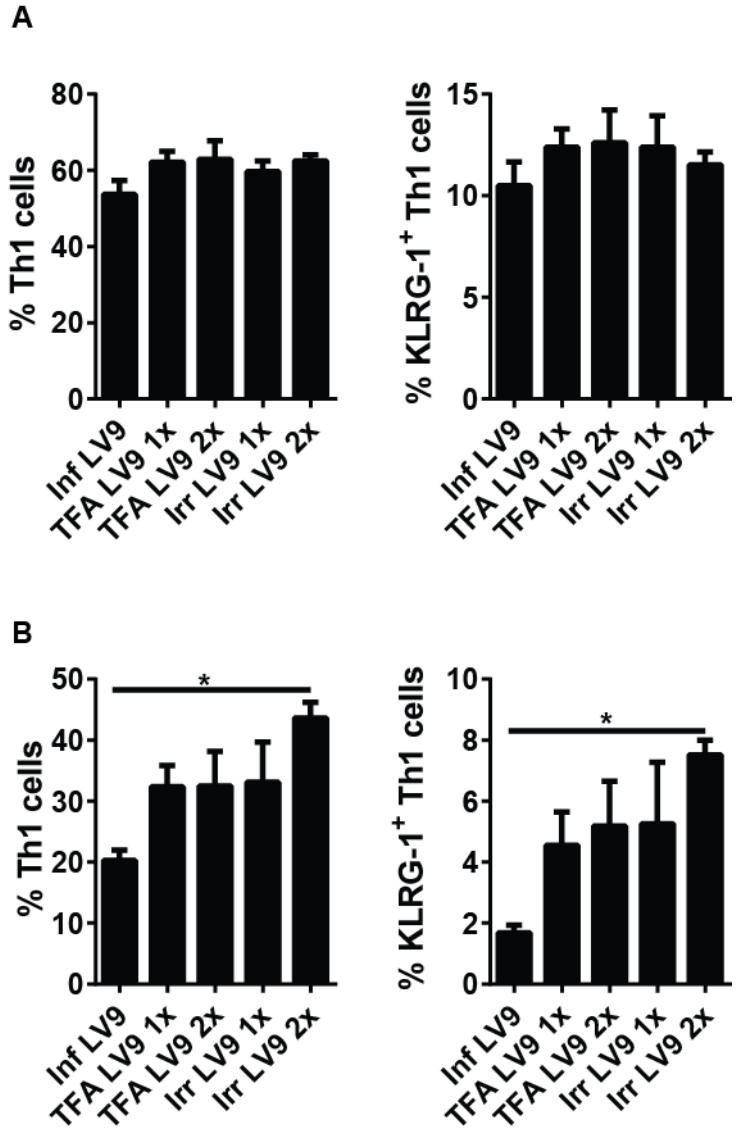

Figure 6.4: Immunization has little effect on the CD4$^+$ T cell responses in the liver, but increased CD4$^+$ T cell responses were observed in the spleen.

Hepatic (**A**) and splenic (**B**) Th1 and KLRG-1$^+$ Th1 cellular responses, as indicated, were measured in mice immunized and challenged with *L. donovani* amastigotes. The frequency of Th1 and KLRG-1$^+$ Th1 cells are shown graphically. Data are presented as the mean +/- SEM at day 14 p.c. Statistical differences of $p < 0.05$ (*) are indicated (n=5 mice per group). Results are representative of a single experiment.

Immune cell analysis carried out in liver samples showed little difference in the frequency of Th1 cells and KLRG-1$^+$ Th1 cells between the different immunized groups. Analysis of spleen cells, however, showed increases in the frequency of Th1 cells and KLRG-1$^+$ Th1 cells in immunized mice, compared to control groups. This indicated that

while immunized animals had a higher percentage of Th1 cells, the rate of conversion to the functionally exhausted phenotype was higher in the spleen, and may account for the lack of effect of immunization on parasite burden observed in this organ (Figure 6.4 A and B).

6.2.3 Immunization with irradiated promastigotes provides better protection compared to irradiated amastigotes

To assess whether irradiated *L. donovani* promastigotes (Irr LV9 promastigotes) might be a better vaccine than irradiated amastigotes, C57BL/6 mice were immunized with two doses (2x) of irradiated *L. donovani* amastigotes (Irr LV9 AM) or *L. donovani* promastigotes (Irr LV9 PM) (Refer to Figure 6.1.2 for regime). Mice were challenged with live *L. donovani* amastigotes four weeks after the final immunization (day 56), and were sacrificed on day 14 p.c. Naïve AMC were infected with *L. donovani* at the same time as the immunized mice were challenged. Antigen-specific cellular immune responses were also measured after immunization. Briefly, 4 weeks after final immunization (i.e. day of challenge), a cohort (n=5) of mice immunized with Irr LV9 (AM) or Irr LV9 (PM) were sacrificed, splenocytes harvested and cultured with *L. donovani* parasite antigen for 72hrs. Culture supernatants were then used to analyse antigen-specific cytokine production. Splenocytes from naïve mice were used as controls.

Mice immunized with either Irr LV9 (Am) or Irr LV9 (Pm), but not infected, showed no change in liver or spleen weights, compared to naïve mice (Figure 6.5 A). Measurement of antigen-specific immune responses showed increased levels of the pro-inflammatory cytokines IFNγ and TNFα, and the anti-inflammatory cytokine IL-10, in both immunized

groups, although mice immunized with Irr LV9 (Pm) showed significantly higher levels of these cytokines (Figure 6.5 B).

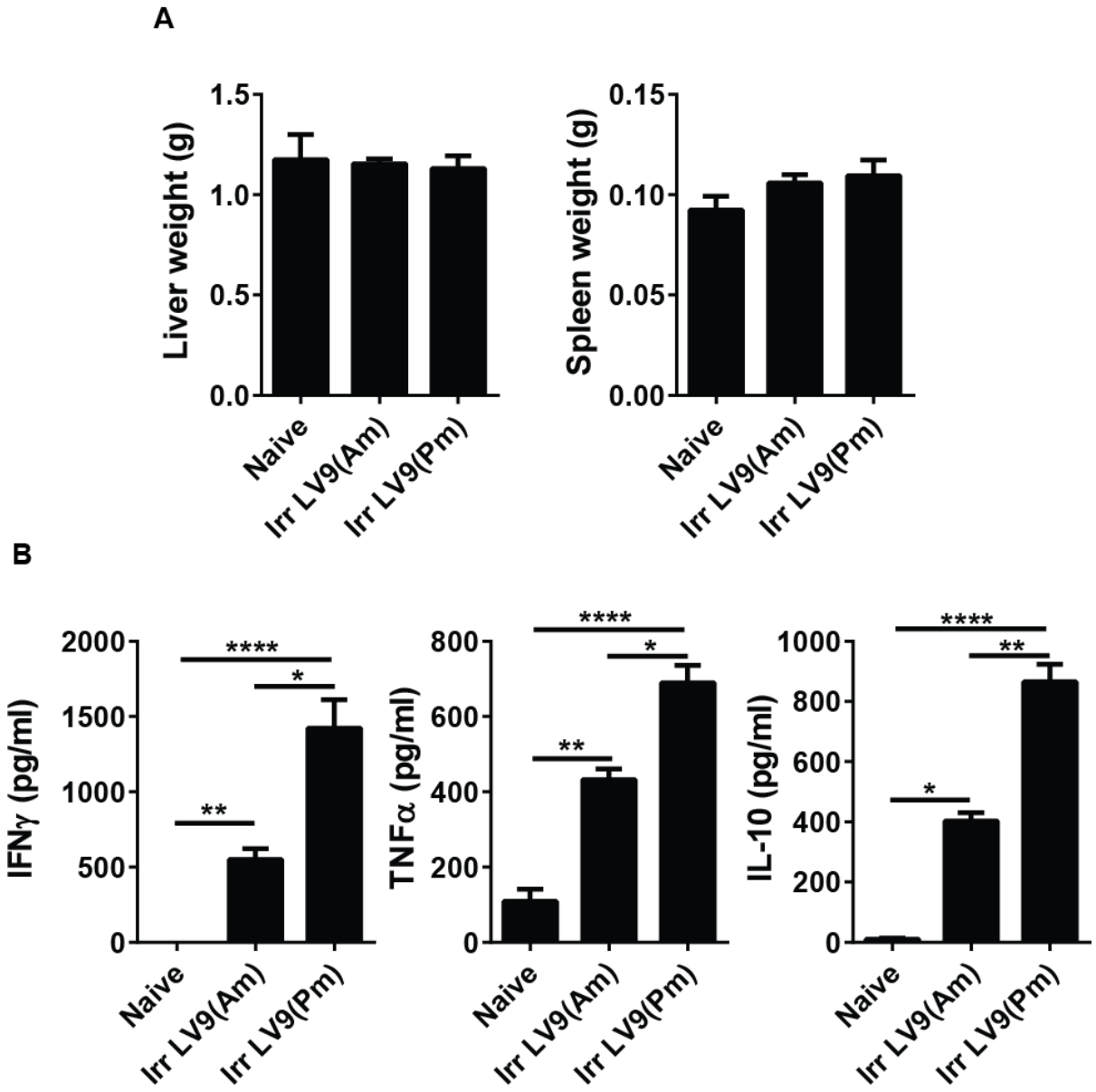

Figure 6.5: Immunization with irradiated parasites results in enhanced antigen-specific T cell responses.

(**A**) Livers and spleens weights were measured in mice immunized with two doses of Irr LV9 (Am) or Irr LV9 (Pm) four weeks after the last immunization. Naïve, non-immunized mice were used as controls. (**B**) IFNγ, TNFα and IL-10 (pg/ml) levels from an antigen-specific re-stimulation assay were measured. Data are represented as the mean +/- SEM on the day of challenge. Statistical differences of $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) are indicated (n=5 mice per group) (NOTE: Re-stimulation assay was performed in triplicate for biological replicates (n = 5/group). Results are representative of a single experiment.

At day 14 p.c., there was a significant increase in the organ weights of mice immunized with two doses of either Irr LV9 (Am) or Irr LV9 (Pm), compared with non-immunized mice (Figure 6.6 A). Assessment of liver parasite burdens showed a significantly lower burden in mice immunized with either Irr LV9 (Am) or Irr LV9 (Pm) (Figure 6.6 B). However, in the spleen, although no change in parasite burden was observed in mice immunized with Irr LV9 (Am), mice immunized with Irr LV9 (Pm) showed significantly lower parasite burdens compared to the AMC group (Figure 6.6 B). Serum cytokine analysis revealed elevated levels of the pro-inflammatory cytokines IFNγ and TNFα in both groups of immunized mice (Figure 6.6 C). These results indicate that immunization with irradiated LV9 promastigotes (Irr LV9 Pm) provides better protection than irradiated LV9 amastigotes (Irr LV9 Am).

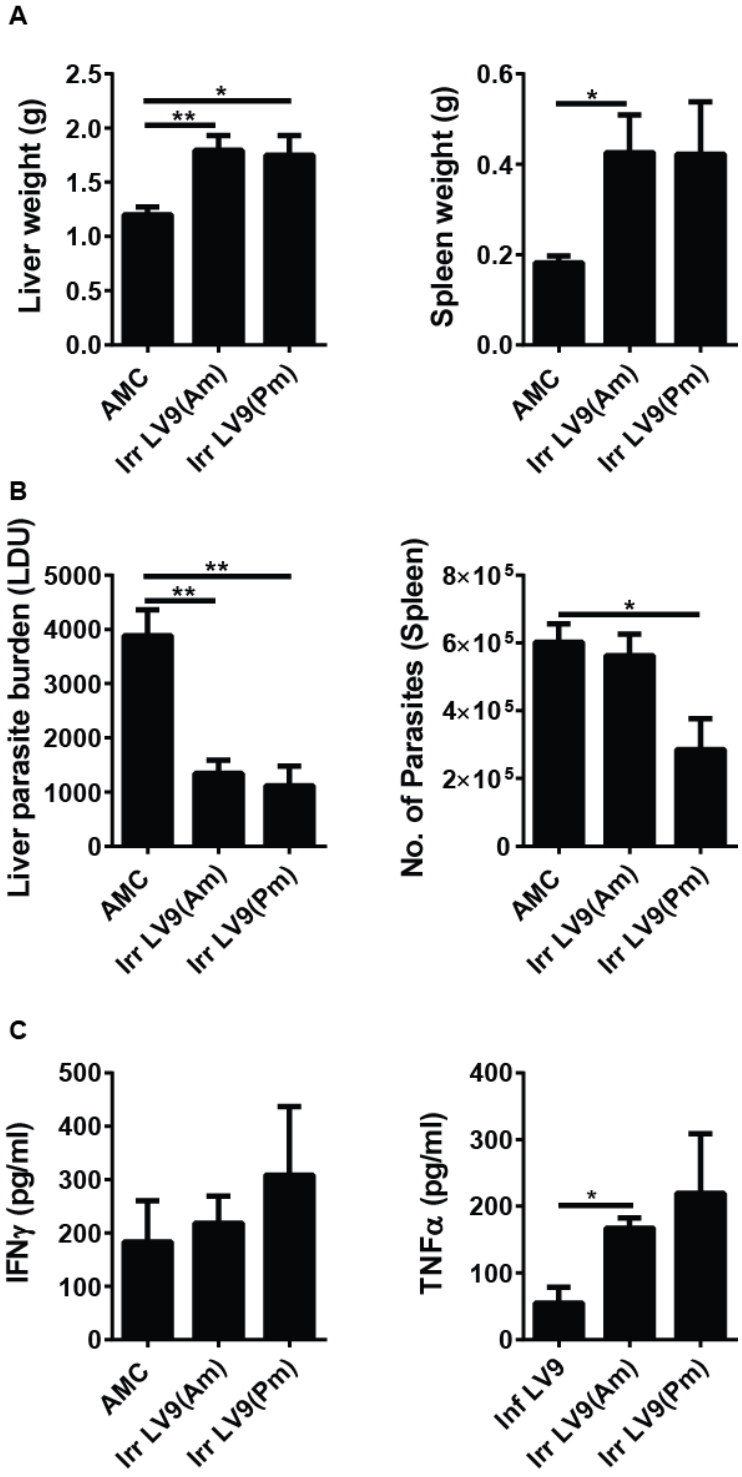

Figure 6.6: Immunization with two doses of irradiated *L. donovani* promastigotes resulted in lower parasite burdens in both the liver and spleen.

Organ weight (**A**) and parasite burdens (**B**) were determined in the livers and spleens of mice immunized with two doses of Irr LV9 (Am) or Irr LV9 (Pm). Mice were challenged with *L. donovani* four weeks after the last immunization and sacrificed on day 14 p.c. AMC were infected with *L. donovani* on day of challenge. (**C**) Serum IFNγ and TNFα levels were measured at the same time. Data are presented as the mean +/- SEM at day 14 p.c. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

I next examined the frequency of Th1 (Tbet$^+$ IFNγ-producing CD4$^+$ T cells) cells and terminally differentiated Th1 (Tbet$^+$ KLRG-1$^+$ CD4$^+$ T cells) cells in different immunized groups (refer to previous chapters for representative gating strategy).

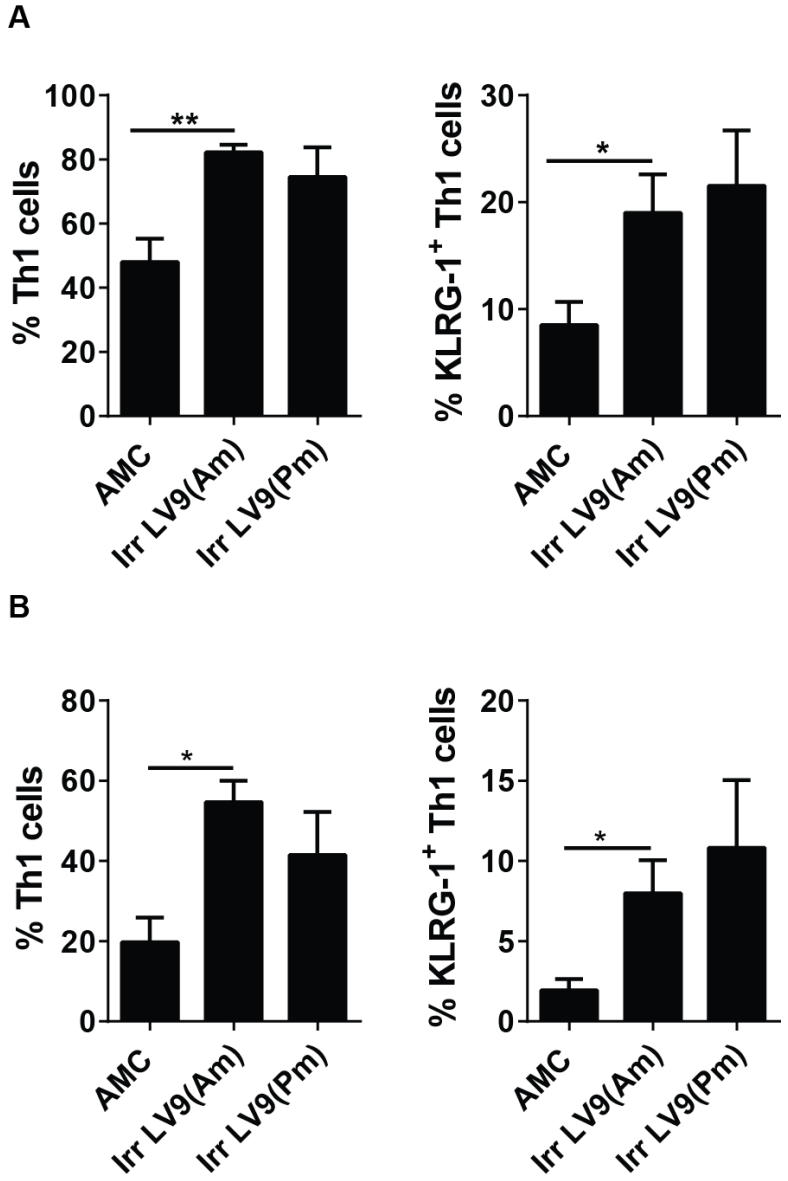

Figure 6.7: Cellular immune responses in the livers and spleens of mice immunized with irradiated parasites and challenged with *L. donovani*.

Hepatic (**A**) and splenic (**B**) Th1 and KLRG-1$^+$ Th1 cellular responses were measured in mice immunized and challenged with *L. donovani*. The frequency of Th1 and KLRG-1$^+$ Th1 cells are shown graphically. Data are presented as the mean +/- SEM at day 14 p.c. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

Analysis of immune cells carried out on liver and spleen cells showed a significant increase in the frequency of Th1 cells in immunized mice, compared to control groups (Figure 6.7 A and B). However, the frequency of KLRG-1^{+} Th1 cells was also significantly increased in immunized animals. This indicated that while immunized animals had a higher percentage of Th1 cells, the rate of conversion to a functionally exhausted phenotype was also higher (Figure 6.7 A and B).

6.2.4 Testing the pathogenicity of irradiated whole parasite

Given the significant splenomegaly observed in mice immunised with 2 doses of irradiated amastigotes, I was concerned that parasites may not have been fully attenuated. Therefore, I next tested whether irradiated LV9 amastigotes could grow in immune compromised mice. B6.Rag-$1^{-/-}$ mice lack mature B and T lymphocytes [246], and were infected with a single dose of either Irr LV9 or TFA LV9 amastigotes. A separate cohort of B6.Rag-$1^{-/-}$ mice were infected with untreated *L. donovani* and 14 days later, groups were assessed for hepatic parasite burdens. Splenic parasite burdens were not assessed since I only wanted to detect establishment of infection.

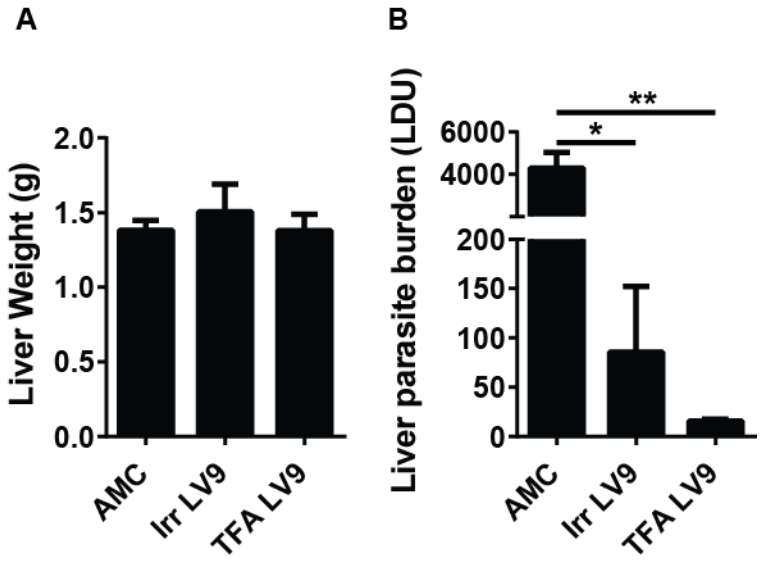

Figure 6.8: Irradiated parasites caused low grade infections in B6.Rag-1$^{-/-}$ mice.

Hepatic weight (**A**) and parasite burdens (**B**) were determined in the livers of B6.Rag-1$^{-/-}$ mice infected with Irr LV9 or TFA LV9 (1x) and sacrificed on day 14 p.i. Parasite burdens in B6.Rag-1$^{-/-}$ mice infected with the same number of untreated *L. donovani* (AMC) were also measured. Data are presented as the mean +/- SEM at day 14 p.i. (n= 5 mice per group). Results are representative of a single experiment.

B6.Rag-1$^{-/-}$ mice infected with Irr LV9 or TFA LV9 amastigotes had lower parasite burdens, compared to *L. donovani* infected AMC. However, mice infected Irr LV9 amastigotes had higher parasite burden compared to mice infected with TFA LV9 amastigotes (Figure 6.8 A and B), suggesting some expansion in parasite numbers in the former group.

6.2.5 Investigating the effect of increasing the dose of irradiation for attenuation in immunized mice

The previous results suggested that the dose of radiation being used was not sufficient to fully attenuate *L. donovani*. My dosing (500 Gys) was based on previous work by Scott

and colleagues [243], but earlier work by Howard and colleagues showed that irradiating promastigotes at higher dose (1500 Gys), also resulted in protective immunity [247]. Hence, I examined the impact of increasing the radiation dose from 500 Gys to 1500 Gys. The immunization regime followed is represented in Figure 6.1.2.

Immunized mice showed no increase in organ weights on day 14 p.c. as previously observed (Figure 6.9 A). Significantly, no change in liver and spleen parasite burdens was observed in immunized mice (Figure 6.9 B). Furthermore, there was no increase in serum levels of the pro-inflammatory cytokines IFNγ and TNFα in immunized mice (Figure 6.9 C). Together, these results indicated that immunization with LV9 promastigotes irradiated with 1500 Gys (Irr LV9 Pm) did not offer protective immunity against challenge.

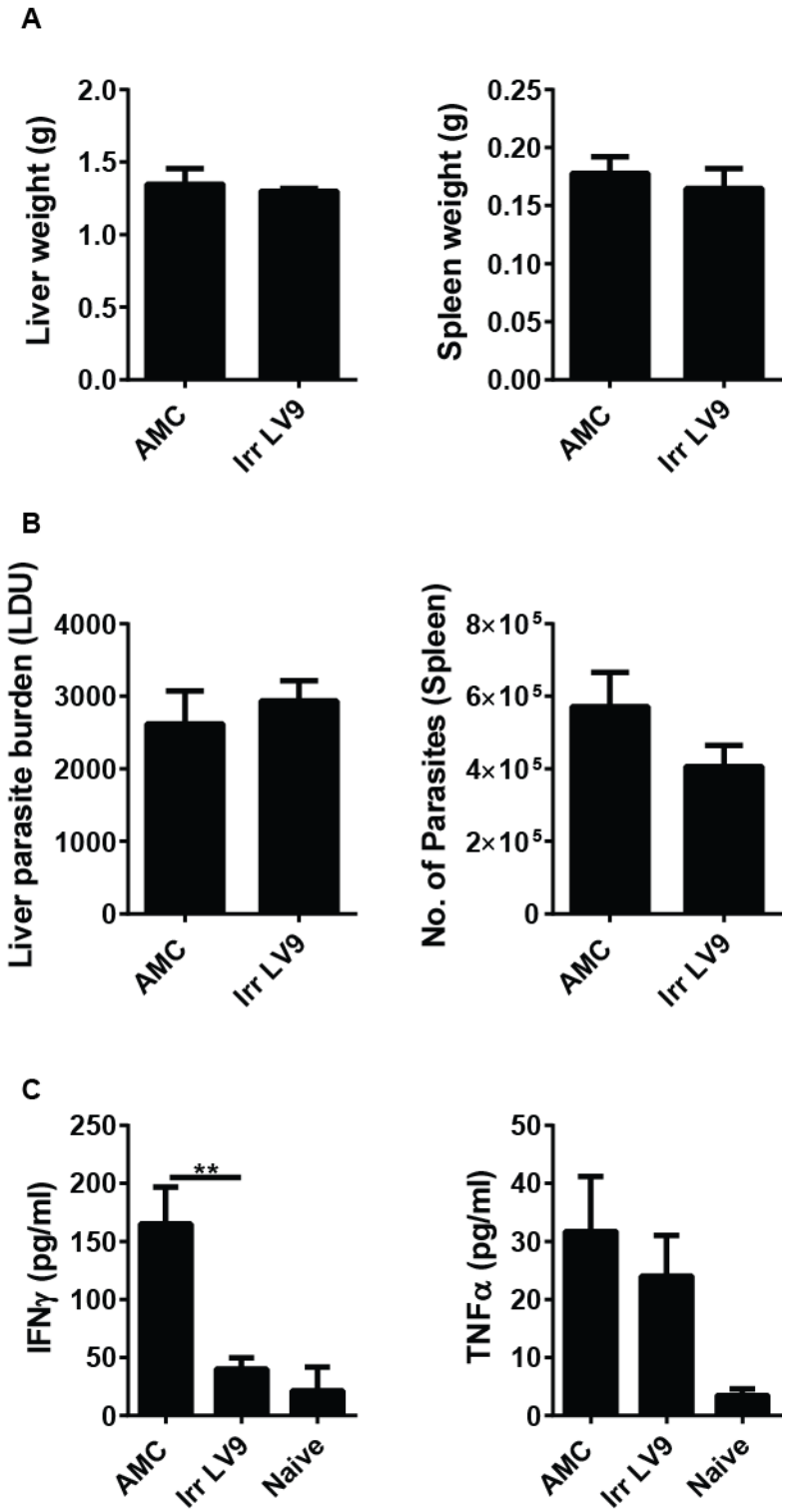

Figure 6.9: Attenuation with a higher dose of radiation results in a loss of protective immune responses.

Organ weight (**A**) and parasite burdens (**B**) were determined in the livers and spleens of mice immunized with two doses of Irr LV9 Pm prepared by using 1500 Gys. Mice were challenged with *L. donovani* four weeks after the last immunization and sacrificed on day 14 p.c. AMC were also infected with *L. donovani* on the day of challenge. (**C**) IFNγ and TNFα levels in the serum of AMC, immunized and naïve control mice were measured. Data are represented as the mean +/- SEM at day 14 p.c. Statistical differences of $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

In order to better understand the above result, an *in vitro* assay was performed using LV9 promastigotes attenuated with 500, 1000 and 1500 Gys to measure the uptake of parasites and production of nitrite (NO_2^-) by macrophages. LV9 promastigotes fixed in 4% paraformaldehyde (PFA) were used as controls.

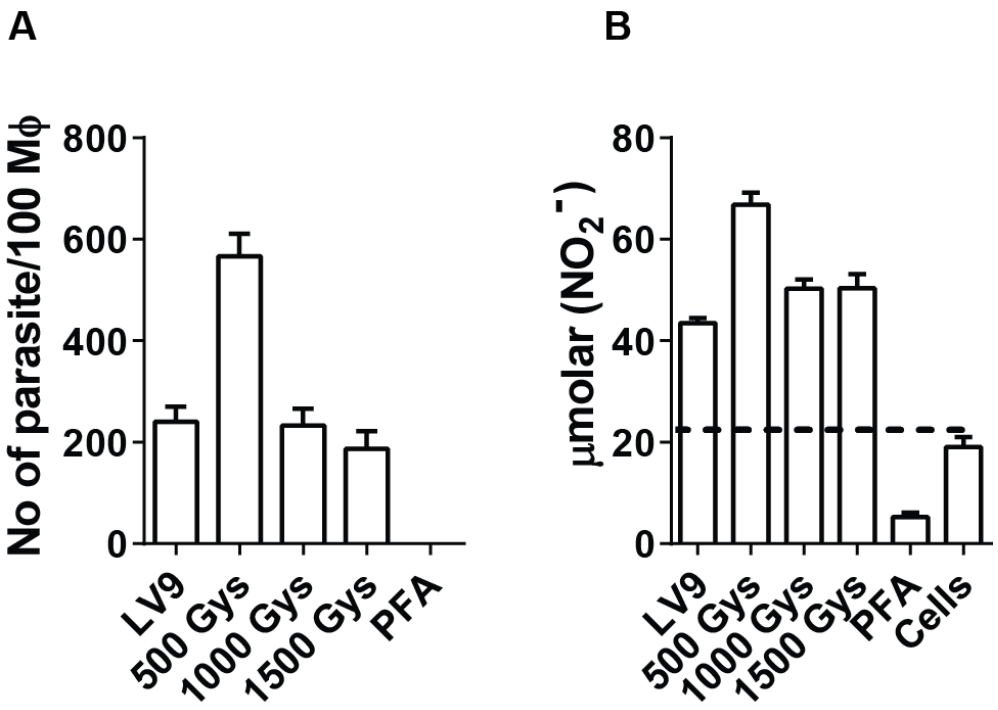

Figure 6.10: Dose of irradiation used for attenuation determines parasite viability and metabolic activity.

(A) Effect of irradiated LV9 Pm on the infection of peritoneal macrophages (Mφ) from C57BL/6 mice. (**B**) Nitrite (NO_2^-) production by C57BL/6 mice peritoneal macrophages (Mφ) 24 hours post culture setup. Data are represented as the mean +/- SEM at 24hrs. (NOTE: Assay was done in triplicate). Results are representative of a single experiment.

The assay revealed that *L. donovani* promastigotes irradiated with 500 Gys were better phagocytized by macrophages, compared to parasites irradiated with 1000 or 1500 Gys (Figure 6.10 A). Also, NO_2^- production was higher in macrophage cultures with parasites irradiated with 500Gys (Figure 6.10 B). PFA-fixed parasites were not able to infect

macrophages and the level of NO_2^- produced by macrophages exposed to these parasites was minimal. These results suggest that parasites irradiated at doses higher than 1000 Gys were less viable and metabolically active, compared to parasites irradiated at 500 Gys, and this may offer some explanation for the results in the previous experiment. However, despite the increased ability of *L. donovani* promastigotes irradiated with 500 Gys to be phagocytosed and induce NO_2^- production by macrophages, these parasites do not appear to be fully attenuated (Figure 6.8 A and B). Hence, based on these results I decided to abandon using radiation as a means of attenuating parasites, and instead focus my efforts on chemical attenuation of *L. donovani* promastigotes for development as a vaccine candidate.

6.2.6 The effect of immunization with chemically-attenuated *L. donovani* promastigotes in the presence of adjuvant

To assess if immunization with chemically-attenuated *L. donovani* promastigotes (TFA LV9) improved parasite control, C57BL/6 mice were immunized with two doses (2x) of TFA LV9. Mice were challenged with live *L. donovani* amastigote four weeks after the final immunization (day 56) and were sacrificed on day 14 p.c. Naïve AMC mice were infected with *L. donovani* at the same time as the immunized mice were challenged (Figure 6.1.3). Antigen-specific cellular immune responses were also measured after immunization.

Administering an adjuvant as part of the vaccine can increase its ability to induce protective immune responses. Most adjuvants are TLR (toll-like receptor)-agonists and several studies have demonstrated the efficacy of TLR agonists (TLR3 agonists: Poly I:C (polyriboinosinic: polyribocytidylic acid), TLR4 agonists: MPL (Monophosphoryl Lipid),

TLR7 agonist: imiquimod and TLR9 agonist: CpG-DNA (immunostimulatory oligodeoxynucleotides)) against *Leishmania* and several other diseases [248-251]. Therefore, I examined the effect of immunizing with chemically-attenuated promastigotes with and without adjuvant. I focused on using CpG-DNA and Poly I:C, since previous work suggested that these adjuvant are potent TLR-agonists. A single dose of CpG-DNA or Poly I:C, was given along with the primary immunization (Figure 6.1.3).

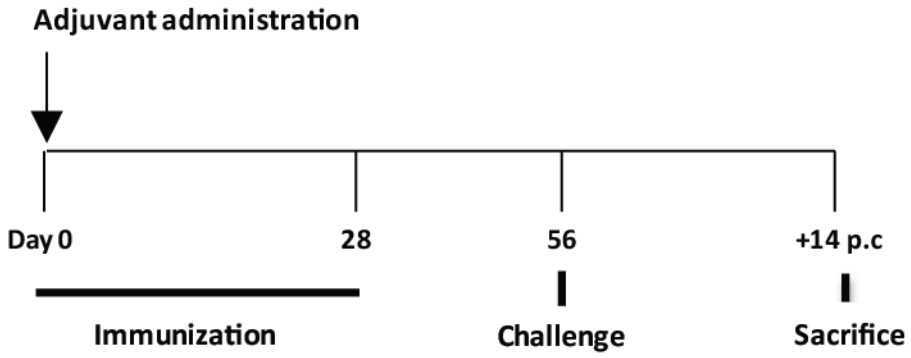

Figure 6.1.3: Experimental timeline for assessment of chemically attenuated parasites for protection against *L. donovani*.

C57BL/6 mice were immunized with two doses of *L. donovani* promastigotes that were chemically-attenuated with TFA ($2x10^7$ parasites i.v.). The primary dose was given on day 0 and subsequent boost on day 28. CpG-DNA or Poly I:C was administered with the first immunization. Four weeks after the final immunization (i.e., day 56), mice were challenged with $2 x10^7$ live LV9 amastigotes and were sacrificed on day 14 p.c.

Mice immunized with TFA LV9 (Pm) alone or with CpG-DNA or Poly I:C, showed no significant increase in liver or spleen weights, compared to naïve mice (Figure 6.11 A). Analysis of antigen-specific immune responses after immunization, but prior to challenge, showed increased levels of the pro-inflammatory cytokines IFNγ and TNFα, and the anti-inflammatory cytokine IL-10, in all immunized groups (Figure 6.11 B).

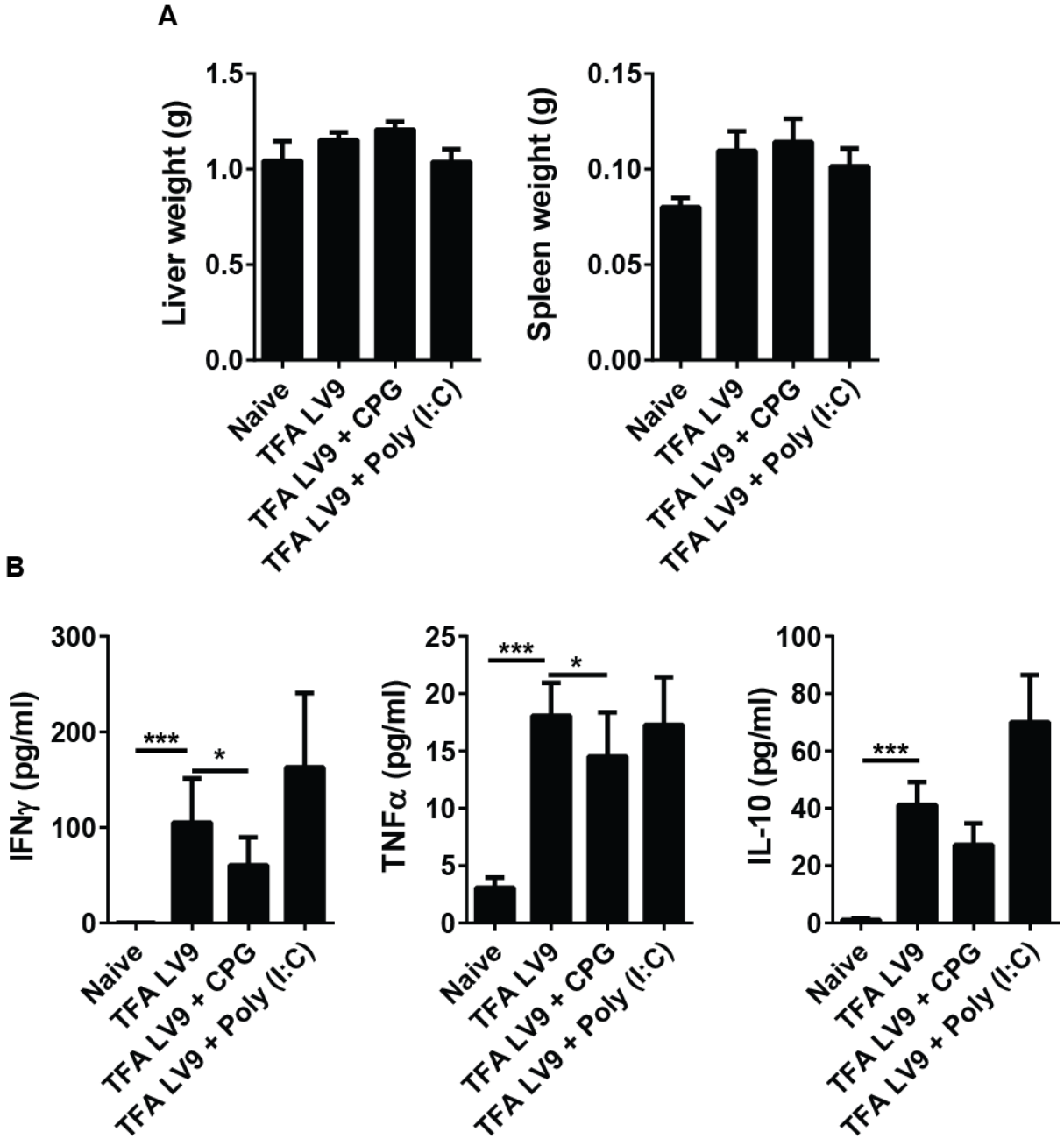

Figure 6.11: Immunization with chemically-attenuated parasites results in enhanced antigen-specific cellular responses.

(**A**) Liver and spleen weights in mice immunized with two doses of TFA LV9 (Pm) with or without CpG-DNA or Poly I:C four weeks after the last immunization. Naïve mice were used as controls. (**B**) IFNγ, TNFα and IL-10 (pg/ml) levels from an antigen re-stimulation assay performed on splenocytes four weeks after the last immunization, but before challenge. Data are represented as the mean +/- SEM at day of challenge. Statistical differences of $p < 0.05$ (*) and $p < 0.001$ (***) are indicated (n=5 mice per group) Results are representative of a single experiment. (NOTE: the antigen restimulation assay was performed in triplicate).

Mice immunized with two doses of TFA LV9 (Pm) alone or with the CpG-DNA showed a significant increase in liver and spleen weights at day 14 p.c. (Figure 6.12 A). However, liver parasite burdens were significantly lower in mice immunized with TFA LV9 (Pm) alone or with Poly I:C, while mice immunized with TFA LV9 (Pm) and CpG-DNA had lower liver burdens, although these were not significantly different from controls (Figure 6.12 B). In the spleen, although there was a trend for lower parasite burdens in all immunized groups, none were significantly different from controls (Figure 6.12 B). Hence, addition of CpG DNA or Poly I:C as adjuvants for chemically-attenuated *L. donovani* promastigotes, did not improve vaccine efficacy.

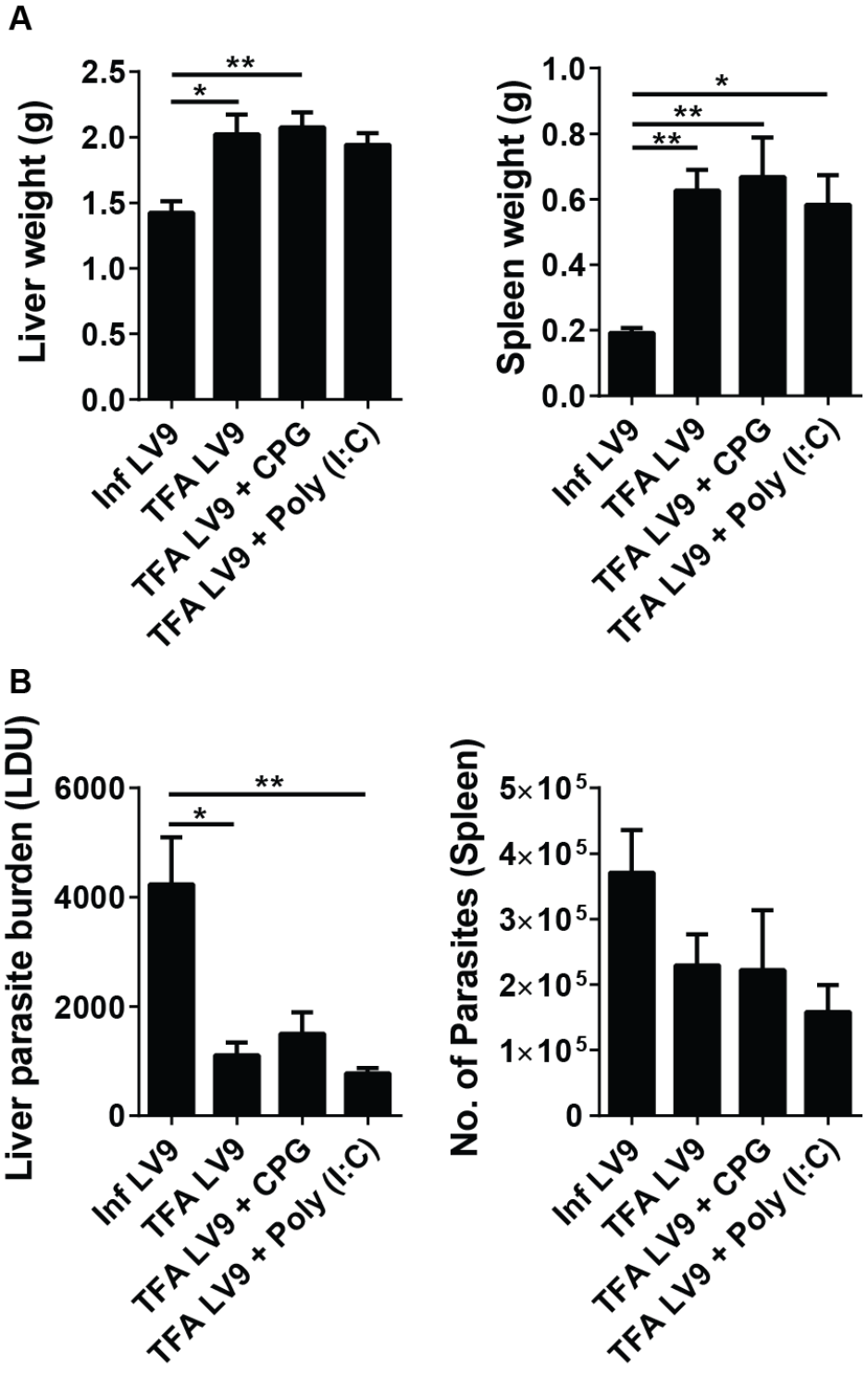

Figure 6.12: Immunization with chemically-attenuated whole *L. donovani* promastigote results in lower parasite burden in both the liver and spleen, but the addition of adjuvant had no effect.

Organ weight (**A**) and parasite burdens (**B**) were measured in the livers and spleens of mice immunized with TFA LV9 (Pm) alone or in combination with CpG-DNA or Poly I:C. Mice were challenged with *L. donovani* four weeks after the last immunization and sacrificed on day 14 p.c. AMC mice were infected with *L. donovani* on the day of challenge. Data are represented as the mean +/- SEM on the day of challenge. Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

I next assessed the $CD4^+$ T cell responses in groups of mice immunized with TFA LV9 (Pm) alone, by examining the frequency of Th1 ($Tbet^+$ IFNγ-producing $CD4^+$ T cells) cells and terminally differentiated Th1 ($Tbet^+$ $KLRG\text{-}1^+$ $CD4^+$ T cells) cells (please refer to previous chapters for representative gating strategy).

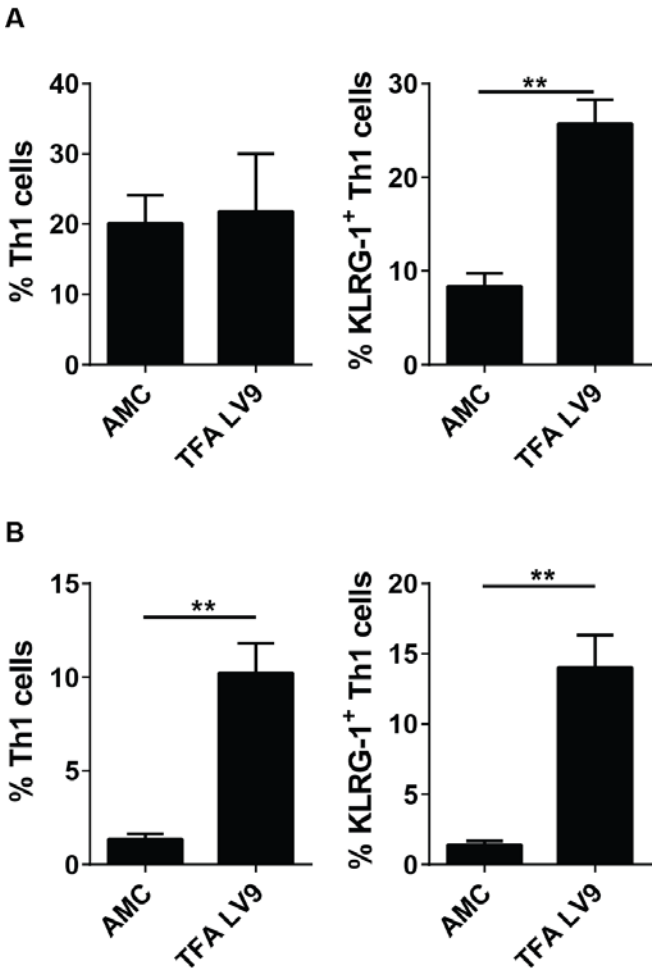

Figure 6.13: $CD4^+$ T cell responses in the livers and spleens of mice immunized with chemically-attenuated promastigotes and challenged with *L. donovani* .

Hepatic (**A**) and splenic (**B**) Th1 and $KLRG\text{-}1^+$ Th1 cellular responses were measured in mice immunized and challenged with *L. donovani* . The frequency of Th1 and $KLRG\text{-}1^+$ Th1 cells are shown graphically. Data are represented as the mean +/- SEM at day 14 p.c. Statistical differences of $p < 0.01$ (**) are indicated (n=5 mice per group). Results are representative of a single experiment.

In the liver, I found no significant difference in the frequency of Th1 cells, but a significant increase in KLRG-1^+ Th1 cells in mice immunized with chemically-attenuated LV9, relative to AMC mice (Figure 6.13 A). In the spleen, a significant increase in the frequency of both Th1 and KLRG-1^+ Th1 cells was observed in groups immunized with chemically-attenuated LV9 (Figure 6.13 B). Thus, a significantly increased Th1 response was only found in the spleens of vaccinated mice, but an increased frequency of functionally exhausted Th1 cells was observed in both tissue sites examined. Together, these data indicate that mice immunized with two doses of chemically-attenuated LV9 promastigotes over a period of eight weeks are partially protected in the liver against an *L. donovani* challenge infection. However, the addition of CpG-DNA or Poly I:C to the vaccine regime did not improve vaccine efficacy. Furthermore, despite an increased frequency of Th1 cells in the spleens of vaccinated mice, this organ remained susceptible to infection.

6.2.7 Chemically-attenuated parasites selectively inhibit pattern recognition receptors

The innate immune system is the host's first line of defence against pathogens. It is activated via pattern recognition receptors (PRRs) including TLRs and NLRs (nucleotide-binding domain like receptors) that are present on and in APCs, where they identify pathogen-associated molecular patterns (PAMPs). From the above experiment, I found that immunizing mice with chemically-attenuated parasite along with adjuvant did not further boost protective immune responses. I therefore hypothesised that chemically-attenuated *L. donovani* parasites inhibited PRR signalling pathways, thus preventing immuno-stimulatory effects of the TLR-agonist CpG-DNA and Poly I:C. Thus, I set up an experiment using purified DC's isolated from the spleens of naïve C57BL/6 mice and cultured them with CpG-

DNA or Poly I:C alone. In addition, chemically-attenuated parasites (TFA treated *L. donovani* promatigotes) were also added to these cultures. I then measured the levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-12 and TNFα in the culture supernatants as these cytokines play important roles in DC maturation and function during infection [252-254].

Levels of the pro-inflammatory cytokines IL-1β, IL-6, IL-12 and TNFα did not change when TFA LV9 was added to unstimulated DC cultures. Although the addition of CpG-DNA or Poly I:C to DC cultures resulted in increased production of IL-1β, IL-6, TNFα and IL-12 (IL-12 levels only increased in the presence of CpG-DNA), the introduction of TFA LV9 caused a significant decrease in IL-1β, IL-6, and TNFα levels, but no change to IL-12 levels. Together, these results show that chemically-attenuated *L. donovani* promastigotes selectively inhibited cytokine production stimulated by stimulation of PRRs (Figure 6.14).

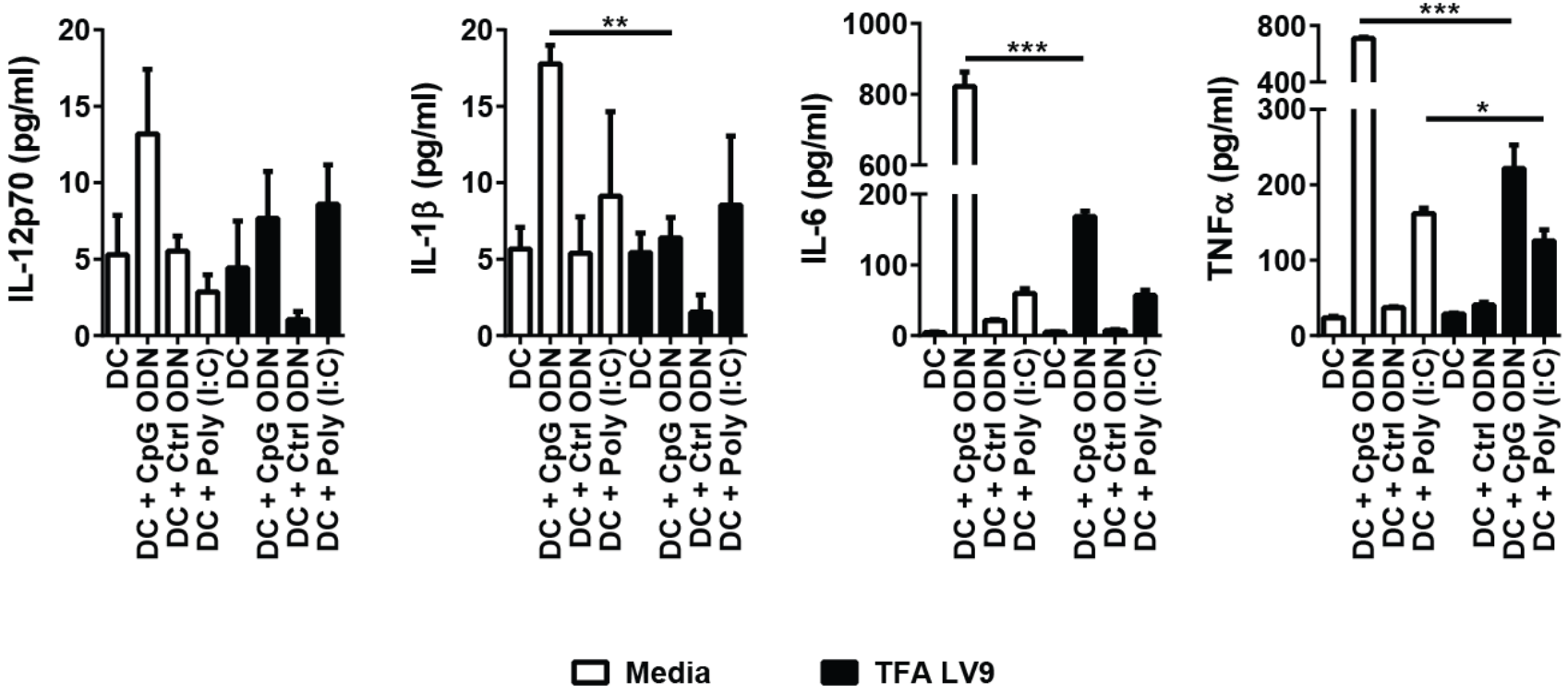

Figure 6.14: Chemically attenuated *L. donovani* promastigotes inhibit pattern recognition pathways in DC's.

IL-12p70, IL-1β, IL-6 and TNFα (pg/ml) levels from the DC cultures. Data are represented as the mean +/- SEM. Statistical differences of $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) are indicated (DCs isolated from n=5 mice and pooled) (NOTE: each cell culture condition was performed in triplicate wells). Results are representative of a single experiment.

6.3 **DISCUSSSION**

In this chapter, I examined the effectiveness of attenuated, whole parasite vaccines, to generate anti-parasitic immune responses against *L. donovani*. To accomplish this, whole *L. donovani* amastigotes or promastigotes were irradiated or chemically-attenuated and used as vaccine candidates. “Leishmaniszation”, the practice of inoculating people with live *L. major* on unexposed areas of the body, resulted in long-term protection against CL infection [236]. This indicates that a vaccine against *Leishmania* is achievable, and I therefore tested whether attenuated whole *L. donovani* parasites could be used to generate protective immunity.

Initially, I aimed to use irradiated *L. donovani* promastigotes as my vaccine following a vaccine regime describe by Scott *et al.* [243]. However, I found little indication that the immunization with different doses of irradiated *L. donovani* promastigotes given through different immunization routes had any statistically significant effect on parasite burdens post challenge. Hence, these results indicated that either the vaccine candidates or the vaccination regime were not ideal. A study by Castiglione *et al.* [255], indicated that the interval between the prime and the boost vaccinations affects the immune response generated. Indeed, I found that parasite burdens were better controlled in the livers of challenged mice when irradiated or chemically-attenuated *L. donovani* amastigotes were used, but the interval between the prime and the boost injection had to be increased from one week to four weeks. However, I discovered that when irradiated *L. donovani* was injected into immune compromised mice, parasite numbers expanded indicating they were not fully attenuated. Increasing the radiation dose, however, lead to the loss of vaccine protection, suggesting that higher doses of radiation caused parasites to be non-viable and unable to induce a potent immune response.

Therefore, I focused my efforts on testing chemically-attenuated *L. donovani* promastigotes, and found they could be used to reduce parasite burdens in the livers of immunized mice, following challenge with *L. donovani*. However, attempts to combine adjuvant with the attenuated parasites failed and no improvement in immunity was observed. Further testing indicated that the live, chemically attenuated parasites were inhibiting pattern recognition pathways on DC's, supporting observation by others with live parasites [256, 257]. I only tested the effects of parasites on CpG DNA- and Poly I:C-mediated activation, so cannot be sure whether all pattern recognition pathways are affected or if this is a selective process. Given the importance of adjuvants for vaccine efficacy, this is an important question that will have to be addressed in the future.

An ideal vaccine is one that elicits strong protective immune responses without causing disease. Achieving the correct level of attenuation is a challenging task, as it is likely to have an impact on the efficacy and safety of the vaccine. If the attenuation is inefficient, there is a chance of parasite growth, resulting in disease progression. If the level of attenuation is too high (e.g. too high a radiation dose), my data indicates this will result in a poor vaccine because the parasites are unable to activate innate immune responses in the host. Manna *et al*. reported that 100-150 Gy was an appropriate radiation doses for parasite attenuation to stimulate an immune response without parasite outgrowth, while 50 a dose of Gy allowed parasite growth [258]. My results showed that attenuation with 500 Gy induced immune responses, while increasing the dose to 1500 Gy resulted in loss a of protective immunity. However, immune compromised experimental hosts infected *L. donovani* parasites irradiated with 500 Gy had higher parasite burden compared to mice infected with the chemically attenuated parasites. Thus, careful attention must be paid to select both the right method and level of attenuation when using live parasites as vaccines.

Vaccines can either be used for prevention of disease or therapeutically. A preventative vaccine could be deployed in VL endemic areas, and also used to protect individuals travelling to these areas. Therapeutic vaccines can be used to treat people with an established infection, and are generally designed to boost the body's immune response in order to better control the infection [259]. One example where a therapeutic vaccine might be useful to control *L. donovani* infection is to help treat post kala azar dermal leishmaniasis (PKDL), which develops after treatment of visceral leishmaniasis (VL) and is observed in 50-60% patients in Sudan and in about 0.8-8% of cases in India [260]. Most PKDL patients heal naturally within 6 months, but those who don't often require months of daily drug treatment. In these patients, a vaccine that boosted anti-parasitic immunity may be helpful. In VL endemic regions, there are many asymptomatic individuals [185]. These people are potential reservoirs of parasite that may contribute to parasite transmission. As such, therapeutic vaccines may be used to reduce parasite loads in these individuals in an effort to reduce the risk of transmission. In an era where VL elimination is on the WHO agenda, it is doubtful that chemotherapy alone will achieve this goal. Therefore, new strategies, such as a therapeutic vaccination, with or without drug treatment, may be useful by reducing transmission of parasites and preventing the development drug resistance in parasites by reducing reliance on chemotherapy.

In summary my study provides proof-of-principal for a chemically attenuated whole parasite vaccine. I identify several key factors that impact on the effectiveness of an attenuated whole parasite vaccine, including the means of parasite attenuation, the vaccination regime, and the adjuvant used. Clearly, there is still much work to be done to develop a chemically attenuated vaccine for testing in humans, but my work has made an important start in this direction.

Chapter 7: Concluding remarks

The main aims of this thesis focused on testing new ways to improve sub-optimal CD4+ T cell responses during *L. donovani* infection. The first two results Chapters (Chapters 4 and 5) of this thesis focused on improving anti-parasitic $CD4^+$ T cells responses generated during experimental VL by using combination immune therapy and cytokine therapy. The third results Chapter (Chapter 6) dealt with generating anti-parasitic immune responses using a whole parasite vaccine.

In Chapter 4, I showed that combined immune therapy with an agonistic anti-GITR mAb and anti-IL-10R blocking mAb had no additive effect on the control of parasite burden in experimental VL, compared to IL-10R blocking alone. However, when mice infected with a lower dose of parasites were treated with the same combination of mAbs, GITR activation actually reversed the positive anti-parasitic effects observed with anti-IL-10R mAb treatment in the liver. This indicated that treatment outcomes depended on several factors, such as parasite load at the start of treatment and the organ being targeted for treatment. Furthermore, our Indian collaborators showed that GITR activation in combination with IL-10 blockade had no significant impact on parasite growth in spleen samples or at improving antigen-specific IFNγ production in whole blood from VL patients (unpublished data; appendix 1). These results suggest that selecting an immune modulator without causing harm to the host is critical. Haque *et al*. [114] showed that stimulation of GITR via an agonistic mAb resulted in expansion of conventional $CD4^+$ T cells. Interestingly, although treatment with the combined mAb treatment resulted in expansion of Th1 $CD4^+$ T cells, these cells had a functionally exhausted phenotype. This highlights the importance of understanding the mechanism of

GITR activity in experimental VL so that the appropriate time to administer anti-GITR mAb can be determined. Antimonial chemotherapy has been the primary line of VL treatment for more than 50 years [3]. However, administration of the drugs over a long period of time resulted in toxicity and drug resistance, and increasing cost of alternative drugs is a major issue. Combining immune modulation that stimulates the immune system with anti-parasitic drugs has the potential to aid in the treatment of a broad range of diseases. In the study by Haque *et al*. mentioned above, administering the agonistic GITR mAb with a suboptimal dose of anti-leishmanial drug resulted in enhanced parasite clearance. In my study, treatment with the combination cocktail along with the drug in a low dose infection resulted in significantly enhanced anti-parasitic immune responses and reduced parasite burdens. Interestingly, IL-10R blockade combined with chemotherapy also resulted in significantly enhanced anti-parasitic immune responses. Studies have shown that targeting PD-1, LAG-3 and CTLA-4 inhibitory receptors individually or in combination in VL, and various other disease settings, have significantly improved immune responses [191, 196, 261, 262]. Further studies should be undertaken in order to establish an ideal combination of immune modulators that can work well with existing drugs to optimise the generation of anti-parasite immunity in VL.

In Chapter 5, I showed that IL-2/anti-IL-2 mAb complex therapy with either IL-2Jc or IL-2Sc significantly reduced parasite burdens in experimental VL. However, an associated expansion of potential target cell populations was not observed. Several studies have shown that $CD4^+$ $Foxp3^+$ T (Treg) cells play a central role in regulating anti-parasitic immunity during *Leishmania* infection, but this is dependent on the parasite species being studied. In *L. major* infection, the role of Treg cells seems to be dependent on the genetic background of the host. In BALB/c mice, Treg cells facilitate protection by dampening detrimental Th2

responses [263], while in C57BL/6 infected mice, anti-parasitic Th1 response are suppressed by Treg cells and prevents sterile cure [211]. In a study by Sacks *et al*. further suggested that in an experimental *L. major* infection, large numbers of IFNγ^+ IL-10$^-$ Th1 cells are need relatively early in the infection to achieve sterile cure [264]. Another study by Ehrlich, A. *et al*. showed that in an experimental *L. (V.) panamensis* infection, induction of Treg cells *in-vivo* with IL-2Jc lead to resolution of active disease [265]. Treatment with IL-2Jc was carried out i.p. over a nine day period (every third day), and resulted in significant expansion of Treg cells at day 2 post-treatment, followed by a decrease in Treg cell numbers by 8 days post-treatment. This study, as well as other, report a 24 hour half-life for the IL-2/anti-IL-2 mAb complex [266], and demonstrate that increased Treg cell numbers and function are short-lived, despite continuation of IL-2/anti-IL-2 mAb complex treatment [210]. This could explain the failure to detect any cellular expansion in my study, and suggests that optimising the dose and timing of IL-2/anti-IL-2 mAb complex (IL-2Jc) treatment may offer a way to improve efficacy. Further investigation into the role of IL-2/anti-IL-2 mAb complexes in promoting the expansion of specific T cell subsets during *L. donovani* infection is warranted.

As mentioned previously, treatment with IL-2Sc resulted in expansion of CD8$^+$ T cells. Stern *et al*. showed that depletion of CD8$^+$ T cells in an established *L. donovani* infection lead to impaired granuloma formation, which resulted in disease exacerbation, thus [65] highlighting the importance of CD8$^+$ T cells for parasite control. In another study by Tsagozis *et al*., isolated antigen-specific CD8$^+$ T cells expressed high levels of IFNγ and TNFα, and elevated cytotoxic activity against *Leishmania* antigens [115]. However, despite the evidence that CD8$^+$ T cells are strong mediators of immune responses against *Leishmania*, recent findings suggest that *L. donovani* induces defective CD8$^+$ T cell antigen-specific responses, by interfering with clonal expansion of CD8$^+$ T cells, resulting in cell dysfunction and

eventually exhaustion [107]. It is again possible that altering the dosing regime for IL-2Sc treatment might better target expansion of anti-parasitic $CD8^+$ T cells, which was not detected in my experiments.

The development of a vaccine to protect against VL depends on generating potent antigen-specific immune responses that are able to protect the host from the primary infection and also provide long-term protection against re-infection. In Chapter 6, I investigated the potential of a live, attenuated whole parasite vaccine against a *L. donovani* infection. I found that vaccine efficacy depended on the interval between the prime and boost, the life cycle stage of the *L. donovani* parasite and degree of parasite attenuation. Mice immunized with the primary vaccine candidate (irradiated *L. donovani* amastigotes), were able to control parasite burdens only in the liver, whereas those challenged with irradiated *L. donovani* promastigotes controlled parasites in both the liver and spleen. However, when irradiated parasites were injected into an immunocompromised mouse, parasites expanded, indicating they were only partially attenuated. Increasing the dose of radiation resulted in loss of protective immune responses conferred by the vaccine, suggesting that the higher dose caused rapid killing of the parasite, and that live, attenuated parasite vaccines should be better than an inactive parasite vaccine. A study by Sacks *et al*. showed that during chronic *Leishmania* infection short lived effector T cells (T_{EF}) are maintained at high frequencies and these are critical for protective immunity against reinfection and mediate a state of concomitant immunity, however these cells fail to survive in the absence of antigen [267]. Developing a vaccine that can provide long term depository of antigen to generate these short lived T_{EF} would be worth investigating. In a study by Belshe R *et al.* immunizing 7,852 children with either live attenuated influenza vaccine or an inactivated vaccine showed 54.9% fewer cases of influenza in the group that received live attenuated vaccine, compared to the group that

received inactivated vaccine [268], providing further support for the idea that live attenuated vaccines will be more effective at generating potent immune responses than killed vaccines. However, the right method and dose of attenuation is essential to prevent the establishment of an infection following vaccination. Chemical attenuation is a feasible alternative to radiation, as conditions for attenuation can be strictly controlled. Indeed, we observed that chemical attenuation of *L. donovani* promastigotes controlled burdens in both the liver and the spleen, and although attenuated parasite were detected in immunocompromised mice, it is likely that they derived from the initial inoculum of attenuated parasites. Many studies have shown that administering an adjuvant in combination with a vaccine increases vaccine efficiency [248-251]. In my vaccine studies, however, I found no additive effect of the adjuvants Poly (I:C) and CpG-DNA. A further analysis of this result indicated that chemically attenuated parasites selectively inhibited pattern recognition receptor (PRR) activation of APCs by these molecules. This aspect of a live, attenuated *L. donovani* vaccines needs to be further examined because the vaccine alone does not appear to provide optimal protection. It is possible that adjuvants that target alternative PRR pathways might be better suited to this type of vaccine.

The idea of combining immune modulation with vaccination has been investigated in CL; blockade of IL-10 signalling during vaccination against *L. major* resulted in a significant decrease in inflammation and parasite burden, compared to vaccination alone [269-271]. Based on these and various other studies, it would be interesting to investigate the outcome of combining immune modulating antibodies or cytokine therapy strategies with the attenuated vaccine examined in my study.

In conclusion, my study highlights the following: 1) the need to identify the right targets for immune modulation for a safe and progressive therapy outcome. 2) cytokine therapy with IL-2/anti-IL-2 complexes could be considered as treatment against VL and 3) development of vaccines against VL requires careful consideration, including the type of attenuation, the parasite lifecycle stage, vaccination regime, adjuvants and/or other types of immune modulators.

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Chapter 9: Appendix

9.1 APPENDIX 1

9.1.1 Effect of combination immune therapy on sample obtained from active human VL patients.

To assess potential of GITR as a therapeutic target in human VL, GITR mRNA accumulation on PBMC's from clinical samples before and after drug treatment was measured, this was compared to results obtained from healthy endemic controls (Figure 9.1). Results indicated that GITR mRNA levels increases in VL patients compared to endemic controls and one month post-drug treatment expression decline. Indicating that GITR could be used as a potential target for immune modulation.

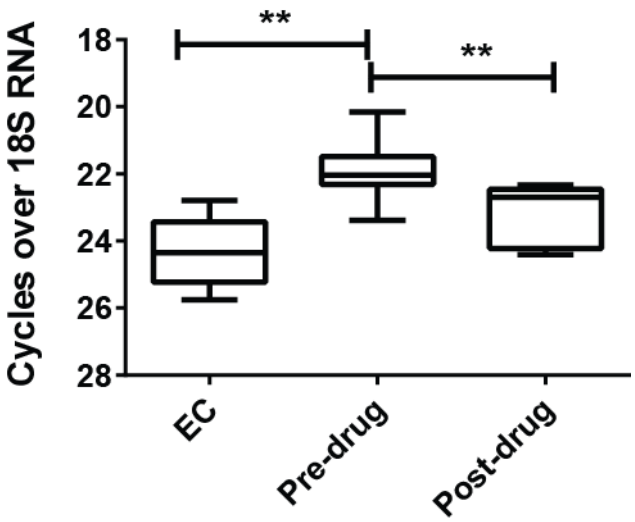

Figure 9.1: GITR mRNA accumulation in PBMC is increased in VL patients.

The relative expression of GITR mRNA in PBMC of VL patients was measured by qPCR before treatment (Pre-drug; n = 7) and 28 days after the commencement of treatment (Post-drug), as well as in healthy endemic control (EC; n = 5) samples. Statistical differences of $p < 0.01$ (**) are indicated.

To determine the effect of immune modulation as a therapy in VL patients, a whole blood assay was carried out. PMBC's from active VL patients were cultured with anti-GITR or anti-IL-10 or a combination of both in the presence of parasite antigen to assess anti-parasitic responses viz IFNγ production. No improvement in the production of IFNγ was observed following the addition of agnostic anti-GITR, blocking of IL-10 resulted in increased IFNγ production, a previously reported result [54] (Figure 9.2). However in cultures that received the combination of antibodies a reverse effect was observed i.e. decrease IFNγ production, indicating that GITR activation had no effect alone and had a negative effect on the ability of antigen activated cells to respond to IL-10 blockade (Figure 9.2).

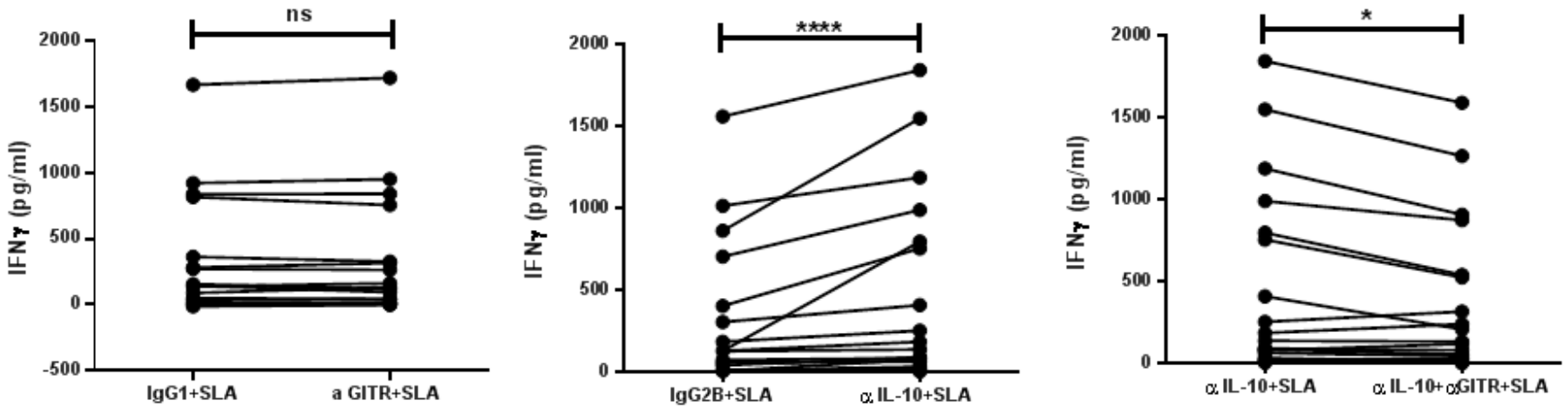

Figure 9.2: GITR activation has no significant impact on parasite growth in spleen samples and antigen-specific IFNγ production in whole blood from VL patients.

A. Spleen cells were cultured in blood agar presence of agonistic anti-GITR mAb or control IgG1, as indicated, before counting the number of viable amastigotes present after 3 days by limiting dilution (n = 15). **B.** Antigen-specific IFNγ production was measured in whole blood cell cultures after 24 hours of stimulation with agonistic anti-GITR mAb or control IgG1, as indicated (n = 19). C. Antigen-specific IFNγ production was measured in whole blood cell cultures after 24 hours of stimulation with a blocking anti-IL-10 mAb, with or without agonistic anti-GITR mAb, and compared with samples treated with control IgG1, as indicated (n = 19). Statistical differences of $p < 0.05$ (*) and $p < 0.001$ (***) are indicated.

To asses if drug treatment could influence combined immune therapy outcome. PBMC'c from active VL patients treated 24 hours prior and 24h hours after drug treatment with a single dose of liposomal amphotericin B (Ambisome^{TM}) were taken. The IFNγ production in responses to parasite antigen was measured in these cells. Prior to drug treatment, anti-IL-10 blockade improved IFNγ production, but activation with agonist anti-GITR supressed the enhanced IFNγ production (Figure 9.3 A). After drug treatment IL-10 blockade improved IFNγ production, addition of anti-GITR had no effect on the level of IFNγ production receiving IL-10 blockade (Figure 9.3 B). Hence these results indicate that although drug treatment reduced the negative effect of GITR activation on IL-10 blockade, targeting GITR as an immune therapy is of no benefit to VL patients over IL-10 blockade.

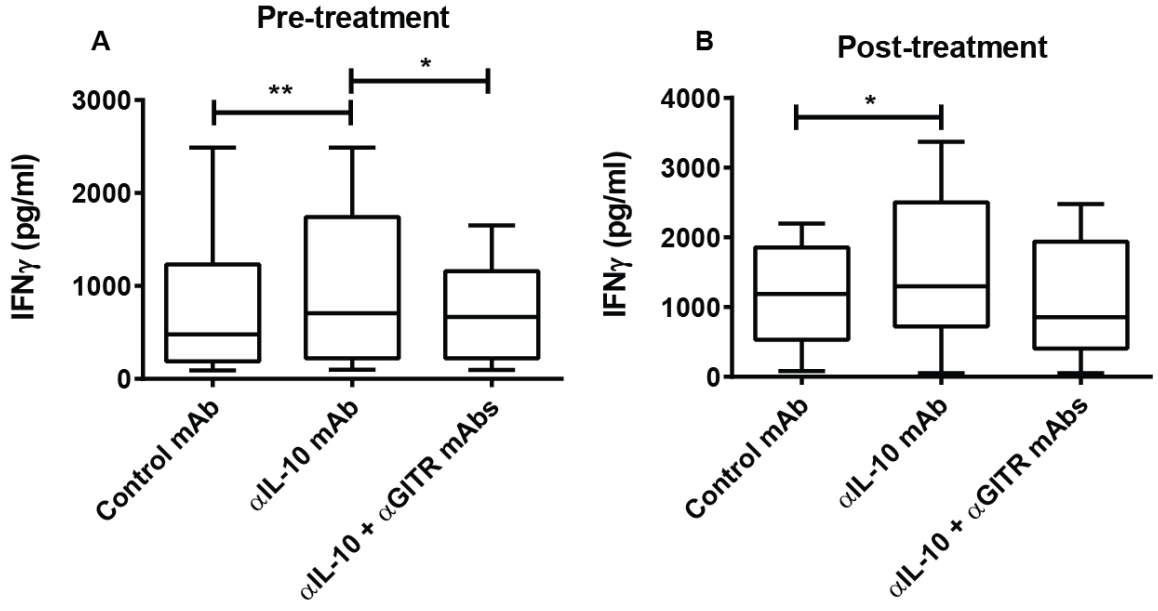

Figure 9.3: GITR activation alone or in combination with IL-10 blockade does not improve antigen-specific IFNγ production by whole blood cells after drug treatment.

Antigen-specific IFNγ production was measured on admission to clinic (**A**), and 24 hours after single-dose ambisome treatment (**B**), in whole blood cells cultured for 24 hours with a blocking anti-IL-10 mAb, with or without agonistic anti-GITR mAb, and compared with samples treated with control IgG1, as indicated (n = 10). Statistical differences of $p < 0.05$ (*) and $p < 0.01$ (**) are indicated.